



# Evaluation of four derivatization methods for the analysis of fatty acids from green leafy vegetables by gas chromatography



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## ABSTRACT

Green leafy vegetables are valuable secondary sources of nutrients, including lipids, commonly consumed in developing countries. However, method development for the analysis of fatty acids is usually focused on the animal lipid samples, rarely including natural plant extracts. Hence, the usefulness of four derivatization methods for the gas chromatographic analysis of plant lipids was studied. Methylation using 10% solution of  $\text{BF}_3$  in methanol and 2.0 M solution of (trimethylsilyl)diazomethane (TMSD) in hexane, trimethylsilylation and *tert*-butyldimethylsilylation were compared using lipid standards and extracts from the leaves of *Solanum macrocarpon* and *S. melongena* after saponification. While silylation was found effective and precise using lipid standards, it initially did not perform well in the analysis of plant lipids due to the presence of transesterification products in samples. Optimization of the hydrolysis conditions resulted in an effective analysis of these derivatives, but poor separation of FA(18:0) from unsaturated FA(18:X) compounds and the presence of larger amounts of interferences disqualified the use silylation for the analysis of plant fatty acids in applied analytical conditions. Methylation using TMSD gave more precise quantitative results when compared to  $\text{BF}_3/\text{MeOH}$  method. Also, it produced a significantly lower amount of interferences when applied to plant lipid samples. Additionally, the TMSD-based method is simple, safe and less time-consuming when compared to other procedures. Thus, we suggest using TMSD-based methylation as a method of choice in the GC analysis of plant-derived fatty acids.

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## 1. Introduction

Tropical green leafy vegetables (GLVs) are consumed widely across Africa and contribute to the more diverse diet [1]. They may also serve as a valuable secondary sources of protein, lipids and microelements [2]. Among GLVs, there are several species belonging to Solanaceae family and genus *Solanum*, including gboma eggplant (*Solanum macrocarpon* L.), which was found to be a relatively rich source of essential amino acids, as well as calcium, copper and magnesium [3]. Recently, the composition of fatty acids (FAs) from raw and blanched leaves of the gboma eggplant was determined [4]. The amount of FAs detected was ca. 2–3% of leaf dry weight, with abundant  $\alpha$ -linolenic acid which accounted for 43–45% of total FAs.

Gas chromatography (GC) is widely used in the analysis of FAs [5]. Lipids extracted from plant or animal tissues are usually hydrolyzed using KOH solution in methanol or ethanol, and FAs released are then subjected to derivatization in order to increase their volatility and decrease polarity of their molecules [5,6]. The most common FA derivatives for the GC analysis are their methyl esters (FAMES). There is a number of reactions used in the synthesis of FAMES: the most common include the use of boron trifluoride in methanol ( $\text{BF}_3/\text{MeOH}$ ), diazomethane and its safer alternative (trimethylsilyl)diazomethane (TMSD). The solution of HCl or  $\text{H}_2\text{SO}_4$  in methanol (HCl-MeOH,  $\text{H}_2\text{SO}_4$ -MeOH) is also widely used in esterification and transesterification of FAs from common lipid classes. However, little is known about the usefulness of certain procedures in the analysis of plant lipid samples. Recently, Alves et al. compared several hydrolysis/derivatization techniques in the analysis of FAs in herbage [7]. They reported quite large differences in the amount of FAs determined, as well as in the amounts of interfering compounds. Most of the common methylation techniques have been used since the late 1950s [8]. They differ in a mechanism of reaction, concentration of reagent used, optimal reaction time

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and possible interferences formed during derivatization [9,10]. More recently, TMSD was proposed as a safe and more stable alternative to diazomethane: it is used mainly for the derivatization of FAs released from animal lipids, including conjugated linoleic acid (CLA) [11–13]. Some reports suggest higher FA recoveries and lower variation of the results obtained using TMSD when compared to the standard acid-catalyzed method [14]. Other methylation techniques used in the analysis of FAs, like in situ transesterification without extraction of lipids [15] or direct transesterification using acetyl chloride [16], are used less frequently. Additionally, derivatives other than FAMES have also been used for the analysis of FAs: these include other alkyl esters [6], as well as trimethylsilyl (TMSi) [17,18] and much more stable *tert*-butyldimethylsilyl (TBDMSi) derivatives [19]. The synthesis of TMSi derivatives may be an useful approach when FAs are analyzed together with sterols [4]. However, their relatively low stability may negatively affect the results [6]. Kloos et al. [20] proposed a sequential one-pot silylation, including the synthesis of TBDMSi derivatives of FAs and then trimethylsilylation of sterols. This method is particularly useful in GC–MS analysis, as TBDMSi esters of FAs give intensive, characteristic fragment ions using electron ionization (EI). Quehenberger et al. [21] proposed the synthesis of pentafluorobenzyl (PFB) esters of FAs, which could be then analyzed using GC–MS and standard non-polar stationary phases.

The comparison of multiple methods of hydrolysis and derivatization used for the analysis of FAs brings variable results. Ichihara and Fukubayashi [10] compared the relative composition of FAs in fish oil determined using four methods for the synthesis of FAMES: the results were comparable. Amusquivar et al. [22] applied standard Folch extraction procedure followed by transesterification of lipids with acetyl chloride (two-step procedure), as well as direct transesterification without extraction, for the analysis of FAs in blood plasma. The latter technique was found to be less reliable. The same authors also suggested using FA(19:0) as internal standard. Similar results were obtained by Mazalli and Bragagnolo [23]: the two-step procedure with  $\text{BF}_3/\text{MeOH}$  as methylation agent resulted in higher amounts of FAMES when compared with direct methylation with  $\text{HCl-MeOH}$ . Antolín et al. [24] synthesized FAMES using  $\text{BF}_3/\text{MeOH}$ , diazomethane,  $\text{HCl-MeOH}$  and  $\text{H}_2\text{SO}_4\text{-MeOH}$ , and TMSi derivatives using *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA). The results of quantitative analyses were similar for all methods; however, when  $\text{HCl-MeOH}$  was used, the reaction time needed was much longer than with use of other reagents. Also, the formation of artifacts during derivatization process depends on the reagent used. Formation of methoxy-substituted FAs during the esterification of unsaturated FAs using  $\text{BF}_3/\text{MeOH}$  was reported by Lough [25]. Park et al. [11] reported that methylation of CLA using  $\text{HCl-MeOH}$  resulted in formation of allylic methoxide, while the use of TMSD could produce TMSi derivatives of CLA. The formation of artifacts during synthesis of TMSi derivatives was broadly reviewed by Little [26]. In our previous report [4], we described the extensive formation of artifacts during the hydrolysis of lipids extracted from dried plant material according to IUPAC procedure [27] and silylation of released FAs. This suggests the presence of matrix-related difficulties in the analysis of plant-derived FAs, as similar effects were not previously reported in the literature. As leaves of GLVs are often sun-dried and stored for the longer period of time [1], the analysis of lipids extracted from dried plant material is – in this case – the most appropriate. Thus, there was a need to evaluate some other hydrolysis and derivatization techniques that could be used in the analysis of lipids from dried plant material. Hence, the objective of the study was to determine the effectiveness of several methylation and silylation methods of FA derivatization, and to apply them for the analysis of lipids extracted from dried leaves of selected GLVs.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Chemical standards: *n*-eicosane, ethyl nonadecanoate, octadecanoic acid [FA(18:0)], 19-methylarachidic acid [FA(20:0)(19Me)], glyceryl trioleate [TG(18:1(9Z)/18:1(9Z)/18:1(9Z))], glyceryl trilinoleate [TG(18:2(9Z,12Z)/18:2(9Z,12Z)/18:2(9Z,12Z))], glyceryl trilinolenate [TG(18:3(9Z,12Z,15Z)/18:3(9Z,12Z,15Z)/18:3(9Z,12Z,15Z))], glyceryl tripalmitate [TG(16:0/16:0/16:0)] and GLC-10 FAME mix were purchased from Sigma–Aldrich Poland (Poznań, Poland). Derivatization agents: *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) with 1% *tert*-butyldimethylchlorosilane (TBDMC), 10% solution of  $\text{BF}_3$  in methanol and 2.0 M solution of (trimethylsilyl)diazomethane (TMSD) in hexane were also obtained from Sigma–Aldrich Poland (Poznań, Poland). Other chemicals were all analytical grade and were purchased from local distributor (POCH S.A., Gliwice, Poland).

### 2.2. Derivatization procedures

The effectiveness of FA derivatization procedures (trimethylsilylation using BSTFA, *tert*-butyldimethylsilylation using MTBSTFA, methylation using 10% solution of  $\text{BF}_3$  in methanol and 2.0 M solution of TMSD in hexane) was determined using known and equal amounts (0.0585 mg) of 19-methylarachidic acid and *n*-eicosane as internal standard. Stock solutions of both compounds in methylene chloride were used. Each synthesis was performed at 30, 60 and 90 °C, and the reaction time was 10, 30 and 60 min. Five independent samples were used for each derivatization procedure at each combination of temperature and reaction time. Nine combinations of reaction time and temperature and the total of 45 samples were then used for each derivatization agent. Methylene chloride was removed from samples in a gentle stream of nitrogen prior to synthesis. All reactions were performed in 1.5-mL screw-capped glass vials except the synthesis of FAMES using  $\text{BF}_3/\text{MeOH}$ , which was performed in 4-mL vials.

TMSi and TBDMSi derivatives were synthesized by adding 100  $\mu\text{L}$  of BSTFA with TMCS and MTBSTFA with TBDMC, respectively. Then, samples were directly GC-FID analyzed as specified in Section 2.5. The synthesis of FAMES using  $\text{BF}_3/\text{MeOH}$  was performed by adding 100  $\mu\text{L}$  of the derivatization agent solution. After the reaction, 1 mL of water was added to the solution, and FAMES were extracted twice using 2 mL and 1.5 mL portions of *n*-hexane. Then, the solution was dried with  $\text{Na}_2\text{SO}_4$  and concentrated to ca. 0.5 mL in a stream of nitrogen. The synthesis of FAMES using TMSD was performed by adding 125  $\mu\text{L}$  of methanol, 50  $\mu\text{L}$  of toluene and 50  $\mu\text{L}$  of TMSD solution. After the reaction, solvents were evaporated to dryness in a stream of nitrogen and redissolved in *n*-hexane. Then, FAMES were GC-FID analyzed as specified in Section 2.5.

### 2.3. Hydrolysis of standard triacylglycerols

To evaluate the usefulness of each derivatization procedure in the analysis of FAs liberated from the complex lipids, the hydrolysis of triacylglycerols (TGs) was performed. Stock solutions of TGs listed in Section 2.1 and ethyl nonadecanoate as internal standard were prepared in methylene chloride. Equal amounts (0.25 mg) of each compound were taken for the hydrolysis. The mixture was placed in a 4-mL screw-capped glass vial and the solvent removed in a stream of nitrogen. Samples for the further silylation were hydrolyzed with 0.5 mL of 0.5 M KOH in 99.8% ethanol, while those for the synthesis of FAMES were hydrolyzed with 0.5 mL of 0.5 M

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