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Short communication

Evaluation of five methods for derivatization and GC determination of a mixture of very long chain fatty acids ($C_{24:0}$–$C_{36:0}$)

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Abstract

D003 is a new active ingredient consisting of a mixture of very long chain saturated fatty acids ($C_{24:0}$–$C_{36:0}$) in a definite proportion, which shows antioxidant, antiosteoporotic, antiplatelet and cholesterol-lowering effects in experimental models. Five derivatization methods for determining these fatty acids by gas chromatography (GC), using diazomethane, sulphuric acid–methanol, hydrochloric acid–methanol, boron trifluoride–methanol and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide were evaluated. GC analysis was carried out using a BPX-5 wide-bore column and 1-nonadecanoic acid ($C_{19:0}$) as internal standard. Methods were similar on account of the fatty acid content determined (84.2–86.6%). However, whereas the hydrochloric acid–methanol method needed 90 min to complete the derivatization, the other methods only required 10 min. Considering costs, speed, safety and GC response, the method using sulphuric acid–methanol was found the most appropriate for determining these fatty acids. The validation of this method: linearity over a range 40–160%, accuracy assessed through a recovery study, precision within day and inter-day, and specificity, even for samples subject to stress conditions, proved it is suitable for quality control and stability studies of the very long chain fatty acids composing this active ingredient.

Keywords: D003; Very long chain fatty acids; Derivatization methods; Capillary gas chromatography; Validation

1. Introduction

Although some reports exist about the determination of long chain fatty acids (LFAs, from 12 to 24 carbon atoms) by high-pressure liquid chromatograpy, gas chromatography (GC) is the technique most widely used with this aim. In this sense, LFAs must be converted to convenient volatile derivatives previous to their analysis. There are many derivatization methods for GC, the majority of them will function quite well when care is taken to use properly [1]. Initially, two organisations that mark rules in the analytic methods: Association of Official Analytical Chemists and American Oil Chemists Society, recommended the use of sulphuric acid–methanol reagent [2,3] for preparing fatty acids methyl esters (FAMEs). However, both organisations accepted later the use of the boron trifluoride–methanol reagent [4,5]. Other methods for methylation of LFAs with good results involve the use of hydrochloric acid–methanol [6], and diazomethane [7], whereas, the methods that employ silylating agents are less used [8].

On the contrary, the GC analysis of very long chain fatty acids (VLFAs, higher than 24 carbons atoms) has had a little interest. This is probably because the VLFAs are less common in the human diet, nor have had a pharmacological interest. However, the development of D003 active ingredient, purified from sugar cane (*Saccharum officinarum* L.) wax has caused a turn on this topic. This natural product consists of a mixture of free saturated VLFAs, from 24 to 36 carbon atoms [9], in a definite proportion with cholesterol-lowering, antioxidant, antiplatelet [10,11], and antiosteoporotic effects [12,13].

As part of the chemical characterization and quality control of D003 at research and development stage, appropriate GC analytic methods were validated for the determination of its content of VLFAs [14–16]. To our knowledge, all these methods are the first in which VLFAs are derivatized to FAMEs using the hydrochloric acid–methanol reagent. However, because of the long time consumption of this acid-catalyzed reaction, with the subsequent delay to deliver the quantitative result, these methods were not considered practical for the routine of quality control.

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In this sense, taking into account the experience provided by previous GC works with LFAs, other derivatization methods for the analysis of D003 were studied.

The GC determinations of these VLFAs, after derivatization using diazomethane, *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), sulphuric acid–methanol, boron trifluoride–methanol, and hydrochloric acid–methanol reagents, including a kinetic evaluation of these reactions, are shown in this paper. The validation of the GC method using the derivatization process that was found as more suitable for this active ingredient is also presented.

2. Experimental

2.1. Materials

D003 (batch 990703) was provided by the National Center for Scientific Research (Havana, Cuba); all other chemicals were analytical reagent grade: hydrochloric acid (37%), sulphuric acid (98%), methanol, toluene, ether, boron trifluoride–methanol (14% solution in methanol), hydrochloric acid (0.1 M), hydrogen peroxide (30%), sodium hydroxide (0.1 M), *n*-hexane, chloroform and sodium hydroxide (99%, Merck, Darmstadt, Germany), and MSTFA (Sigma, St. Louis, USA). The diazomethane was generated from *N*-methyl-3-nitro-1-nitrosoguanidine (99%, Riedel-de-Haën, Seelze, Germany) in a mini-diazomethane generator.

Stock solution comprised of tetracosanoic ($C_{24:0}$), pentacosanoic ($C_{25:0}$), hexacosanoic ($C_{26:0}$), heptacosanoic ($C_{27:0}$), octacosanoic ($C_{28:0}$), nonacosanoic ($C_{29:0}$), triacontanoic ($C_{30:0}$) and hentriacontanoic ($C_{31:0}$) acids (Sigma, St. Louis, USA) was prepared as previously described [15].

The nonadecanoic acid ($C_{19:0}$), approximately 99% pure by GC (Sigma, St. Louis, USA), was used as internal standard (IS) at 1 mg ml^{-1} in two solutions, one in chloroform (IS solution A) and another in *n*-hexane (IS solution B). These solutions were found to be stable for at least 1 month when stored at +8 °C.

2.2. Chromatographic conditions

The GC system consisted of a GC-14B with a flame ionization detector (Shimadzu, Kyoto, Japan). A BPX-5 wide-bore fused silica capillary column (25 m, 0.53 mm i.d., 1.0 μm D_f; SGE, Texas, USA) was used, from 220 °C to 320 °C at 5 °C min^{-1} and isothermal for 10 min at 320 °C. Injector and detector were set at 320 °C. Carrier gas (H_2) flow was 11 ml min^{-1}. To form the flame, hydrogen gas flow, 40 ml min^{-1}, and air gas flow, 400 ml min^{-1}, were used.

The GC–Mass Spectrometry system (GC/MS) consisted of a GC 8000 coupled to a MD800 series (Fisons, Manchester, England) with a capillary column SPB-5 (30 m, 0.25 mm i.d. and 0.25 μm D_f; Supelco, Bellefonte, USA). Operating conditions: column programmed from 100 °C to 200 °C at 40 °C min^{-1}, from 200 °C to 320 °C at 10 °C min^{-1} and isothermal for 30 min at 320 °C. Helium carrier gas flow was 1 ml min^{-1}. Injector, ion source, and interface temperatures were 320 °C, 250 °C, and 250 °C, respectively. Ionization energy was 70 eV. The mass spectrum was continuously acquired from 40 to 600*m*/*z* with a scan speed of 1 s/decade in full scan mode.

2.3. Sample preparation

Hydrochloric acid–methanol: 1 ml of the IS solution A was added into a 4 ml vial containing previously 10 mg of D003, then the solvent was evaporated to dryness at 80 °C under a gentle air flow. One millilitre of the methylating reagent (5% aqueous hydrochloric acid–methanol, v/v) was added. The vial was heated at 80 °C with occasional shaking. Afterwards, the sample was evaporated to dryness at 80 °C under a gentle air flow. Then, 1 ml of toluene was added and the vial was again tightly closed and heated at 80 °C for 3 min.

Diazomethane: 1 ml of the IS solution A was added into a 4 ml vial containing previously 10 mg of D003, then the solvent was evaporated to dryness at 80 °C under a gentle air flow. One millilitre of the ethereal diazomethane reagent was added. The vial was left at room temperature. Afterwards, the sample was evaporated to dryness at 45 °C. Then, 1 ml of *n*-hexane was added and the vial was heated at 80 °C for 3 min.

Boron trifluoride–methanol: 1 ml of the IS solution A was added into a 4 ml vial containing previously 10 mg of D003, then the solvent was evaporated to dryness at 80 °C under a gentle air flow. One millilitre of the methylating reagent (methanol containing 14% (w/v) boron trifluoride) and 1 ml of *n*-hexane were added. The vial was heated at 60 °C with occasional shaking. Before the analysis of the *n*-hexane phase the sample was allowed to rest for 5 min.

Sulphuric acid–methanol: 1 ml of the IS solution B and 1 ml of the methylating reagent (2% sulphuric acid–methanol, v/v) were added into a 4 ml vial containing previously 10 mg of D003. The vial was heated at 80 °C with occasional shaking. Afterwards, 0.25 ml of the neutralising aqueous solution (sodium hydroxide at 1 M) was added and it was smoothly shaken. Before the analysis of the *n*-hexane phase the sample was allowed to rest for 5 min.

MSTFA: 1 ml of the IS solution A and 50 μl of MSTFA were added into a 4 ml vial containing 10 mg of D003 and it was heated at 60 °C.

In all cases five reaction times were evaluated and 1 μl portions were analysed by GC.

2.4. Identification and calibration

FAME identification criterion was the relative retention calculated from a D003 sample, which was previously analysed by GC/MS. Quantitative analysis was based on the IS method, previous determination of the relative mass response factor (f_i^m) from samples prepared using the stock and the IS solutions, according to the following equation:

$$f_i^m = \frac{A_{is} \times m_i}{A_i \times m_{is}}$$

where A_{is} is the peak area of the IS, m_i the mass of component i (mg), A_i the peak area of the component i and m_{is} is the mass of IS (mg).