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ABSTRACT: OEA is known to potentially have beneficial biological effects on weight management by controlling food intake and activating lipid catabolism. In biological fluids, OEA and other endogenously biosynthesized FAEAs, are usually analyzed by tandem liquid-chromatography mass-spectrometry (LC-MS). The present study provides analytical method to routinely assess the quality of OEA prepared for biological studies by gas-liquid chromatography (GLC). The preparation of OEA for biomedical studies can be performed by *N*-acylation of oleic acid/esters or using oleoyl chloride. In the present study, OEA was prepared by transamidation of triolein. The analysis of the synthesized OEA has been performed by gas-liquid chromatography of its trimethylsilylether (TMS) derivatives. Free OEA cannot be analyzed as such because dehydration of the ethanolamide moiety promptly happens in the GLC injection. This thermal degradation reaction gives rise to the formation of an oxazoline derivative. The TMS moiety prevents the reaction and the structure of the formed derivative was assessed by mass-spectrometry. We show here, that OEA prepared for biological studies can be routinely analyzed by GLC after TMS derivative preparation.

1. INTRODUCTION

Fatty acid ethanolamides (FAEA) belong to a family of lipids naturally found in both plant and animal tissues. Moreover these fatty acid derivatives appear to have biological properties. Indeed palmitoylethanolamide (derived from palmitic acid) have antinociceptive and anti-inflammatory properties [1]. Among this family, anandamide (derived from arachidonic acid) has been of great interest. In the last decade, it was discovered that anandamide is an endogenous ligand for cannabinoid receptor subtype 1 (CB1). Activating CB1, anandamide increases food intake. Another interesting fatty acid ethanolamides is oleoylethanolamide (OEA), formed from oleic acid and phosphatidylethanolamine in the brain and in the intestine[2-5]. Biological function of OEA such as anorexigenic or body fat loss properties have been extensively studied over the past decade [5-6]. This molecule is naturally present at low concentrations in food products such as cocoa powder (up to 2mg/g), oatmeal or nuts [7]. The OEA biological function is to regulate food intake *via* a synthesis/degradation balance, which occurs mainly in the enterocytes (brush border) [3,8]. The biological mechanism of action including non-genomic effect mediated through peroxisome proliferator-activated receptor alpha (PPAR-α) [9] and transient receptor potential vanilloid type 1 (TRPV1) receptor are discussed [10,11].

LC-based methodologies have been developed to quantify OEA and other endogenously formed FAEAs in biological fluids and tissues [8]. The level of OEA in plasma or other fluids, such as cerebrospinal fluid, is very low (around 10 pmol/ml), but recent development allowed to reach a quantification limit below 1 pmol by LC-MS/MS using electrospray ionization in the positive mode and silver cation coordination [12]. Quantification can also be performed using single stage LC-MS as shown by Giuffrida *et al.* [13], or by RMN analysis [14]. However, LC-MS is not a routine method to assess the quality of OEA preparation used in biological experiments.

OEA have been synthesized from triolein by our research group to conduct chronic oral supplementation in further studies. The objective of the present study was to develop a method to assess the quality of OEA preparation produced for biomedical studies.

2. MATERIALS AND METHODS

2.1. Chemical and reagents. Ethanolamine (99% purity), trimethylsilylchlorosilane (TMCS) and N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Sigma-Aldricht (Saint Louis, USA). Triolein was obtained from Nu Check Prep (Elysian, USA). Sodium sulfate decahydrate was obtained from Riedel-de Haën (Hanover, Germany).

2.2. Synthesis of oleoylethanolamide(OEA) from triolein. OEA was prepared as described by Roe et al. [15] with slight modification. Triolein (1 g) and ethanolamine (0.5 g) were stirred under inert conditions in a test tube and the mixture was heated at 170°C for 30 min. After cooling to room temperature, the residue was dissolved in hexane (25 mL) and washed with warm sodium sulfate (10% aquous solution, 200ml) in a funnel. The organic phase was washed another time with the same solution. The solvent was removed and OEA has been stored at 4°C under argon.

2.3. Preparation of trimethylsilylether (TMS) derivatives. TMS derivative was prepared following the standard AOCS method Cd 11b-91 [16]. Briefly, OEA (10 mg), trimethylchlorosilane (200 µL) and BSTFA were stirred in a test tubes heated at 70°C for 20 min. After, cooling to room temperature OEA TMS derivative was diluted in hexane at 0.1 mg/mL.

2.4. Gas-liquid chromatography analysis. Analyses of OEA TMS derivative and free (OEA 0.1 mg/mL) were performed by GLC using a DB-5HT capillary column (15 m x 0.25 mm i.d., film thickness 0.10 µm; J&W, Palo Alto, USA). Split injection (50:1) and flame-ionization detection (FID) were achieved at 320°C and 400°C, respectively. Oven temperature

programming was 150°C, increased to 300°C at 20°C min^{-1}, and then to 380°C for 2 min at 10°C min^{-1}. Carrier gas (H2) was used in constant flow mode at 2 $ml.min^{-1}$.

2.5. Gas-chromatography mass-spectrometry (GC-MS) analysis. OEA was analyzed by GC-MS as its TMS derivative on a 6890 Series II gas-chromatograph (Agilent, Palo Alto, CA) attached to a 5973N selective quadrupole mass detector (Agilent Technologies, Palo Alto, CA)] under an ionization voltage of 70 eV, and connected to a computer with a Hewlett-Packard ChemStation. The injector, in split mode (25:1), and the interface temperatures were maintained at 250˚C and 280°C, respectively. Helium was used as carrier gas under constant flow rate (1.8 mL min-1). GC separation was performed on a DB-1 capillary column (30 m x 0.25 mm i.d., film thickness 0.25 µm; J&W, Palo Alto CA). Oven temperature programming was 100°C, increased to 270°C at 50°C min^{-1}, isothermal for 12 min. Electron impact mass spectra were recorded in the 100-400 u mass range.

3. RESULTS AND DISCUSSION

Alkanolamides can be synthesized by direct transamidation of alkanolamine with purified fatty acids or methyl esters derived from vegetable oils and or animal fats. FAEAs such as OEA can be produced by a Schotten-Bauman reaction [14] where a fatty acyl chloride is reacted with ethanolamine or according to Roe et al. [15] starting from fatty acid ester such as triacylglycerol. In the present study, we used triolein as a starting material (see **Figure 1**). The reaction is complete after 30 min and need to be performed at 170°C. The GLC method described utilizes a wide range of temperature starting from 60°C to 380°C and can therefore be used to analyze glycerides or fatty acids (results not shown). Therefore, the given conditions can be used to calculate the yield of the reaction. However, using the described parameters used for the synthesis of OEA, remaining traces of triolein, diolein or monoolein were not detected.

Excessive temperature (*i.e.* >200°C) can lead to the formation of a degradation product. This reaction also happens when OEA is analyzed as a free compound by GLC. The degradation product is less polar and elutes before free OEA (see **Figure 2A**). The exposure to high temperature (*i.e.* in the injection port) catalyses the self-condensation of the ethanolamide moiety giving rise to the formation of an oxazoline derivative as described in **Figure 3**. This type of reaction is routinely used to prepare dimethyloxazoline derivatives from fatty acid esters and 2-aminopropanol for mass-spectrometry analysis of fatty acids [17]. In addition, this reaction can be performed using trifluoroacetic anhydride and mild temperature as described by Kuklev and Smith [18]

Free OEA needs therefore to be stabilized prior to GLC analysis. TMS derivative has been prepared and analyzed under the same analytical conditions (see **Figure 2B**). The derivatization reaction was performed according to the AOCS method Cd 11b-91 using TMCS and BSTFA [16]. The structure of the obtained derivative was analyzed by GC-MS. The typical mass-spectrum of OEA as TMS derivative is shown in **Figure 4**. The mass spectrum is characterized by an intense molecular ion at 397.2 amu and a fragment ion at 382.2 amu that corresponds to a loss of a methyl group of the TMS moiety. Only a trace amount degradation product represented less than 1% were detected by GC-MS and a typical GLC-FID chromatogram is shown in **Figure 2B**. This result shows that the derivatization of OEA can effectively be performed using standard procedure and that the obtained derivative is more stable under thermal condition.

3. CONCLUSION

The analysis of OEA by GLC is a convenient technique to analyze the composition of OEA preparation. However, OEA need to be derivatized prior GLC analysis due to its poor thermal stability. The formation of the oxazoline derivative can be prevented by preparing TMS derivative that stabilizes the ethanolamide moiety. This method can be routinely used to

assess the quality of the synthetic preparation produced for biomedical studies. For quantitative analysis of complex FAEA mixtures in biological fluids, the basic principle, described in the present study, can certainly be applied. However, the chromatographic condition and the quantification procedure need to be developed.

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Legend of Figures.

Figure 1. Preparation of oleoylethanolamide (OEA) by transamidation of triolein.

Figure 2. Gas-liquid chromatography analysis of (A) free oleoylethanolamide (OEA) or (B) as its trimethylsilylether (TMS) derivative.

Figure 3. Formation of an oxazoline derivative of oleoylethanolamide (OEA) thermal degradation of at high temperature (>200°C).

Figure 4. Mass-spectrum of oleoylethanolamide (OEA) as trimethylsilylether (TMS) derivative. Ionization performed by electron impact at 70 eV.

Fig 1.

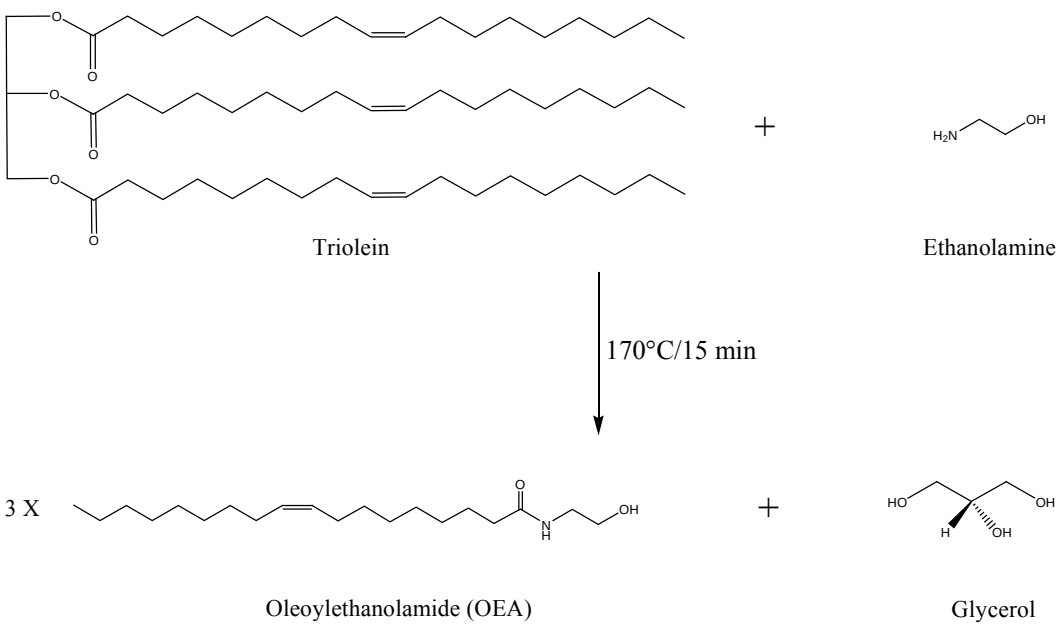

Fig 2.

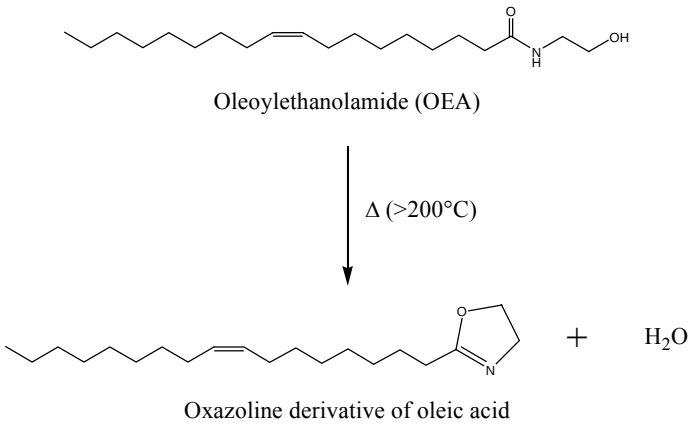

Fig 3.

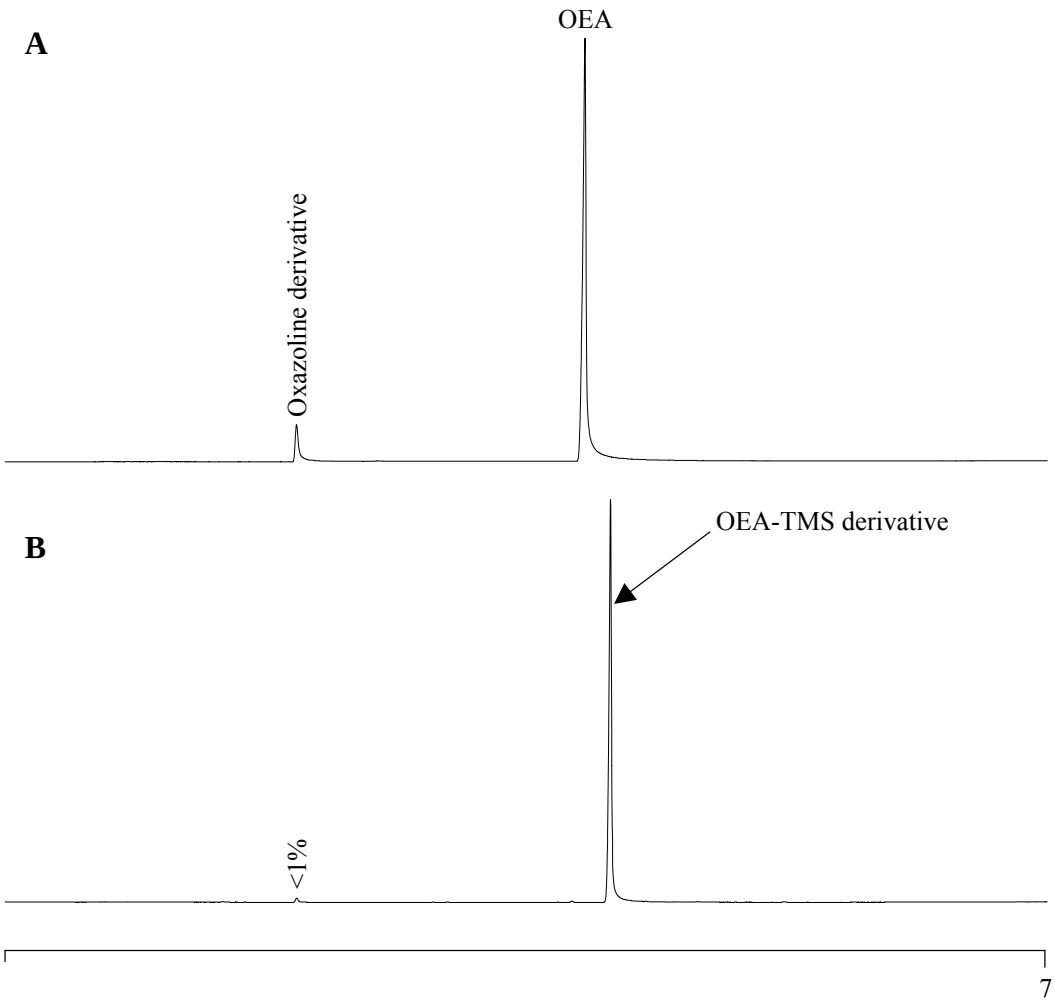

Fig 4.