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HPLC-APCI-MS/MS method development and validation for determination of tocotrienols in human breast adipose tissue

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ABSTRACT

For the last decade, significant attention has been paid to the potential role of tocotrienols in prevention and therapy of breast cancer. Therefore, the aim of this study was to develop and validate analytical method for quantitative determination of tocotrienols (α-, β-, γ- and δ-tocotrienol) in human breast adipose tissue with the use of high performance liquid chromatography coupled with APCI-MS/MS detection. Separation of target compounds was achieved within 10 min with the use of naphthylethyl Cosmosil 2.5π-NAP column with methanol/water mixture (90:10, *v/v*) under isocratic elution. Adipose tissue samples were obtained from breast cancer patients and women deceased as a result of accidents. Sample preparation procedure was optimized with the application of the Plackett-Burman design and included tissue homogenization with the use of isopropanol/ethanol/aqueous 0.1% FA mixture (13:3:8, *v/v*), centrifugation and solid phase extraction (SPE). The method was validated in terms of linearity, precision, accuracy, stability (bench top, autosampler, postpreparative, freeze and thaw stability), matrix effect (ME), recovery (RE) and process efficiency (PE). As for all four tocotrienols ME was negligible (< 15%), precision and accuracy tests were performed with the use of tocotrienols' standard solutions within the ranges of 10.0–400.0 ng/g for all four tocotrienols. As the validation requirements were met, the validated method was applied for quantitative analysis of tocotrienols in breast cancer patients.

1. Introduction

Tocochromanols, namely tocopherols (T) and tocotrienols (T3), belong to a group of eight naturally occurring substances widely known as vitamin E family members [1–3] (Fig. A.1, Supplementary materials). For a few decades, they were reported to present potential activity against cancer diseases, especially breast cancer, in numerous *in vitro* and *in vivo* tests [4–7].

Interestingly, the most promising anticancer tocochromanol properties are attributed to tocotrienols, the unsaturated homologues of tocopherols [2,5]. Apart from being well known potent antioxidants, they were also suggested to exhibit anti-inflammatory [8], neuroprotective [9], antidiabetic [10] and cholesterol-lowering properties, along with protective activity against cardiovascular diseases [11,12]. Recent findings have also proved tocotrienols' antiangiogenic [5,13] and antiproliferative [14,15] activity against human breast cancer cells, which may be further investigated in terms of potential anticancer properties. Since very little is known about the actual quantity of tocotrienols in adipose tissue, development and validation of a sensitive method for their determination is necessary.

Isolation of tocopherols and tocotrienols from the adipose tissue and their subsequent reliable quantification is challenging and in the literature, only a few examples can be found. Shim et al. described a method for quantitative analysis of α- and γ-tocopherols after saponification of breast adipose tissue samples with further extraction of target compounds using ether/hexane mixture [16]. In another example, Gleize et al. implemented Bligh and Dyer extraction and saponification in order to determine α- and γ-tocopherol with other lipid antioxidants in subcutaneous adipose tissue with photodiode array detection [17]. To our best knowledge, only one paper [18] has described simultaneous quantification of tocopherols and tocotrienols in human breast adipose tissue. Nesaretnam et al. [18] described extraction procedure which included homogenization of 500 mg of adipose tissue in hexane/ethanol/0.9% aqueous NaCl (4:1:1, *v/v/v*) mixture.

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The structural similarity of tocol derivatives significantly affects their chromatographic separation. Satisfactory separation of all tocochromanols is easily achieved with the use of normal-phase high performance liquid chromatography [19,20]. However, poor analytical reproducibility and low stability of silica phases directed researchers towards reversed-phase HPLC [21]. Furthermore, as tocols possess natural fluorescence, a considerable part of papers concerning their quantification describes the application of HPLC-FLD methods for this purpose [22–25]. The use of fluorescence detection for the determination of tocopherols and tocotrienols provides high sensitivity; however, achieving lower limits of quantification is always a desirable result [26,27]. In contrast to fluorescence detection and at the expense of problematic ionization of target compounds, mass spectrometry offers advantageous sensitivity as well as selectivity and was therefore implemented in experiments described below.

So far, very few methods for quantitative determination of tocotrienols with the use of reversed-phase liquid chromatography techniques coupled with mass spectrometry (LC-MS) have been described. In general, atmospheric pressure chemical ionization (APCI) is a suitable technique for the analysis of low-molecular-weight compounds of low-polarity. Therefore, it is usually preferred to perform the analysis of tocochromanols in both positive [19,20,28–30] and negative [31,32] polarities. However, only in a few cases validation procedures were performed for tocotrienols' quantification in human plasma [19] and plant food [31]. Nevertheless, Liang et al. developed an ESI-LC-MS/MS method for γ-tocotrienol quantification in rat plasma with lower limit of quantification (LLOQ) equal to 10 ng/mL [33]. In the majority of papers, negative ESI-LC/MS methods were applied for the determination of tocols in biological and plant matrices [26,34–36]. A detailed description concerning tocochromanol analysis with the use of chromatographic techniques coupled with mass spectrometry was discussed in our previous review article [21].

The objective of the study was to develop the analytical method for quantitative determination of α-, β-, γ- and δ-tocotrienol in adipose tissue with the use of LC-MS/MS. The method proposed by our group allows using a limited amount of adipose tissue sample (100 mg) followed by its rapid homogenization and extraction with the use of more environmental friendly solvents, such as isopropanol and ethanol instead of hexane. Moreover, low quantification limits of target compounds (10 ng/g), as well as short time of the chromatographic separation (10 min) with the application of naphthylethyl column stationary phase were achieved in this work. The established method was validated and applied to determine tocotrienols' concentrations in breast adipose tissue from patients enrolled in a clinical study.

2. Experimental

2.1. Chemicals

Analytical standards of tocotrienols (T3) (α-, β-, γ- and δ-), methanol (LC-MS grade) and buthylated hydroxytoluene (2,6-di-*tert*-butyl-4-methylphenol, BHT) were obtained from Sigma Chemical Co. (Sigma Aldrich, St. Louis, MO, USA). Ethanol (HPLC grade) and 2-propanol (LC-MS grade) were obtained from Baker (Avantor Performance Materials B.V., Deventer, The Netherlands). Formic acid (97%) was delivered by Alfa Aesar (A. Johnson Matthy Company, Karlsruhe, Germany). Deionized water was produced with the use of a D-100TUIM water system (Labopol-Polwater, Kraków, Poland).

2.2. Instrumentation and HPLC-MS/MS conditions

Quantitative determination of T3 was performed with the use of Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with a triple quadrupole mass analyzer (Agilent 6430 Triple Quadrupole Mass Spectrometer, Santa Clara, CA, USA). The HPLC system consisted of membrane degasser (G1322A), binary pump (G1312B) and thermostated autosampler (G1329B).

Table 1
MRM ion transitions of tocotrienols (T3) chosen for the study.

Compound	MRM transition[a]	Fragmentor voltage [V]	Collision energy [V]
α-T3	425/165	130	19
	425/205	130	11
β-T3, γ-T3	411/151	135	27
	411/191	135	15
δ-T3	397/137	125	30
	397/177	125	15

[a] Upper and lower panel refer to the quantitative and qualitative MRM transition ions, respectively.

The chromatographic analyses were performed with the use of naphthylethyl column (Cosmosil 2.5π-NAP, 2.5 μm, 3.0 × 100 mm, Nacalai Tesque, Inc., Kyoto, Japan) and methanol/water mixture (90:10, *v/v*) under isocratic elution. The total analysis time, sufficient for the separation of all tocochromanols was 10 min. Column temperature, flow rate and injection volume were set at 20 °C, 0.5 mL/min and 4 μL, respectively. The autosampler was thermostated at 4 °C. APCI source was used under the following conditions: gas temperature: 320 °C, vaporizer temperature: 375 °C, gas flow: 4 L/min, nebulizer pressure: 30 psi, corona current: 4 μA, capillary voltage: 3500 V. The fragmentor voltage parameters and multiple reaction monitoring (MRM) transitions selected with the use of the Mass Hunter Optimizer software (Agilent Technologies, Santa Clara, CA, USA) are summarized in Table 1. Fragmentation pathways of T3 are presented in Fig. 1.

The analyses were carried out with the use of Agilent Qualitative Mass Hunter Workstation (Agilent Technologies, Santa Clara, CA, USA) composed of LC/MS Data Acquisition for 6400 Series Triple Quadrupole version B.07.01, Qualitative Analysis version B.06.00 and Quantitative Analysis for QQQ version B.07.00.

2.3. HPLC-FLD conditions used for experimental design step

In order to optimize sample preparation procedure (tissue homogenization and extraction, solid phase extraction (SPE)) Agilent HPLC 1200 system (Agilent Technologies, Santa Clara, CA, USA), composed of membrane degasser (G1322A), quaternary pump (G1311A), thermostated autosampler (G1329B) and fluorescent detector (G1321B) were used. The excitation and emission wavelengths were set at 298 and 330 nm, respectively.

2.4. Sample collection and storage conditions

Breast adipose tissue samples were obtained from two groups of subjects. Women who underwent a surgical intervention due to the presence of malignant breast cancer were enrolled in the first group and signed an informed consent before entering the study (5 participants). Adipose tissue was obtained from the peripheral area dissected around the breast lump during surgery. The second group of subjects consisted of women deceased in accidents (5 women). In this case breast adipose tissue was dissected up to 24 h after the time of death. The project was approved by the Bioethical Committee of the Medical University of Gdańsk (No. NKBBN 464/2013).

Tissue fragments obtained from oncological surgery and forensic medicine departments were sealed in cryogenic tubes and frozen at – 20 °C. Within a week, samples were transferred to – 80 °C refrigerator for a long-time storage and kept frozen until analysis.

2.5. Sample preparation

Each adipose tissue fragment was thawed on ice for 30 min and