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Fast derivatization procedure for the analysis of phytoestrogens in soy milk by gas chromatography tandem mass spectrometry

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ABSTRACT

In this work an optimized GC–MS/MS method, based on a simple and fast derivatization procedure, is proposed for the determination of five phytoestrogens (formononetin, daidzein, coumestrol, genistein and biochanin A) in soy milk. A systematic study of the instrumental analysis conditions was carried out, optimizing different MS acquisition parameters. At the same time, the derivatization procedure was optimized testing three silylating reagents. Among them, *N*,*O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) was chosen as the most suitable derivatization reagent and an experimental design was performed to find the best conditions for the silylation reaction. The results showed the independence of the derivatization efficiency from the heating treatment applied before analysis, enabling the derivatization directly in the injection port; moreover, the use of a temperature programmed injection enhanced sensitivity and precision of the analysis. The described optimization steps allowed a significant gain in the sensitivity of the overall method, leading to detection limits of 0.1–17.7 μg L^{-1}, which are lower or comparable to values reported in the literature for GC–MS analysis of these analytes. High specificity was reached as well, thanks to the careful study of the conditions for tandem MS detection. Precision was good for formononetin, daidzein and coumestrol, with coefficients of variance of 4.7–6.1%; on the contrary, the analysis of genistein and biochanin A was characterized by low precision, probably due to poor stability of the corresponding derivatives. The method was tested on some soy milk samples from the Italian market, proving its suitability for the analysis of phytoestrogens in this complex matrix.

1. Introduction

Phytoestrogens are a class of natural non-steroidal compounds, widespread in the plant kingdom, that comprises different groups, such as isoflavones and coumestans. For a long time, positive effects have been attributed to phytoestrogens, such as antioxidant activity [1], anti-carcinogenic activity [2,3], protection against cardiovascular disease [4] and alleviation of menopausal symptoms [5]. Nevertheless, due to the proven estrogenic activity, phytoestrogens are considered endocrine disrupting chemicals (EDCs) [6]. In particular, the impact on the reproductive development is concerning when the intake of endocrine disruptors occurs in early childhood, since adverse effects may occur even years later, e.g. during puberty [7]. Furthermore, there are conflicting results regarding the anti-carcinogenic action of phytoestrogens in breast cancer development; some data indicate no positive effects and sometimes even suspected induction of the tumor [8,9].

The major sources of phytoestrogens are red clover, legumes, licorice, hop, alfalfa and, above all, soy. Nowadays the consumption of soy-based food is increasing and a lot of new products are appearing on the market. Since the beneficial or detrimental effects of phytoestrogens on humans are still not completely understood, it is important to determine the phytoestrogens content of soy-based food, to evaluate the intake of these compounds, which may exert positive or negative effects, based on environmental and individual factors (age, physiological state, exposition to other EDCs etc.).

The most used technique for the quantification of phytoestrogens in food is liquid chromatography, usually coupled to mass spectrometry (LC-MS); Kuhnle et al. [10–12] determined the concentration of many phytoestrogens in a wide range of food (including some soy-based foods), combining solid-liquid or liquid-liquid extraction with SPE (Solid Phase Extraction) purification, and analyzing the extracts by HPLC-MS/MS. Other works focused on bovine milk [13] or legumes [14,15].

A few examples of gas chromatography coupled to mass spectrometry (GC–MS) are reported for the analysis of phytoestrogens in food [16,17]. The reason why LC is usually preferred to GC is that for the latter method a derivatization step is necessary. In fact,

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phytoestrogens are slightly polar substances, therefore they are characterized by low volatility and this makes them not suitable for direct analysis by GC–MS.

Nevertheless, GC–MS remains a widespread technique, as well as less expensive in comparison to LC-MS, and it is interesting to test its possibilities in terms of sensitivity and specificity.

The derivatization procedure is a crucial step in this kind of analysis and, as already highlighted in our previous works [18,19], it is not always simple to find the best conditions to make this step fast, reproducible and efficient. The most employed derivatization compounds for phytoestrogens are silylating reagents such as *N*,*O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA), *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MtBSTFA), used in mixtures which include catalysts. BSTFA has been used in combination with 10% of trimethylchlorosilane (TMCS) to perform analysis of phytoestrogens in soy milk and wastewater [17]; derivatization with MSTFA has been applied for their determination in medicinal herbs [20] and estuarine water samples [21]; MtBSTFA with 1% of *tert*-butyldimethylchlorosilane (tBDMCS) has been selected as derivatization mix to analyze phytoestrogens in human urine [22]. In all these cases the derivatization procedure requires heating, incubation time (from 30 min to 4 h) and different steps of evaporation and reconstitution of the solutions; besides, the proposed protocols were obtained by optimizing the involved parameters one at a time.

The aim of this work was to carefully study all the steps involved in the derivatization protocol to attain a new, simple and faster method for the analysis of phytoestrogens by GC–MS. Five of the most commonly studied phytoestrogens were considered: formononetin, biochanin A, daidzein, genistein and coumestrol (shown in Fig. 1). Their derivatization was optimized using different reagents and conditions; the multivariate approach of experimental design was used to rationally plan and perform the experiments. Moreover, a systematic study of the instrumental conditions for the analysis of phytoestrogens by GC coupled to an Ion Trap mass spectrometer was carried out: we tested different methods, changing the instrumental parameters (ion source temperature, tandem mass spectrometry settings, injection conditions) to enhance sensitivity and specificity of the method.

To the best of our knowledge, no similar study of instrumental conditions, nor a multivariate approach to optimize the derivatization, has been reported so far for phytoestrogens. The developed method was validated and applied to the determination of the five phytoestrogens in some soy-based drinks from the Italian market.

2. Materials and methods

2.1. Chemicals and reagents

The phytoestrogens formononetin (FORM, >98%), biochanin A (BIOCH, >98%), daidzein (DAID, >98%), genistein (GEN, ≥98%) and coumestrol (COUM, ≥95%) were purchased from Sigma Aldrich (St. Louis, MO, USA). The derivatizing reagent *N*,*O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA, 99.4%) was obtained from Supelco (Bellefonte, PA, USA) while the derivatizing mixes *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) activated with ethanethiol and ammonium iodide, and *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MtBSTFA, >95%) with 1% of *tert*-butyldimethylchlorosilane (tBDMCS) were from Sigma Aldrich (St. Louis, MO, USA). The catalyst trimethylchlorosilane (TMCS, ≥99%) was obtained from Sigma Aldrich as well and was added to BSTFA to prepare a derivatizing mixture of BSTFA with 10% of TMCS.

Methanol was obtained from VWR Chemicals (Fontenay-sous-Bois, France), dichloromethane was from Lab Scan Ltd. (Dublin, Ireland) and ethyl acetate and pyridine were purchased from Sigma Aldrich (Steinheim, Germany). All solvents were of chromatographic grade. Ultra-pure water was obtained from a Millipore Q-Gard system equipped with a Millipak 0,22 μm filter (Millipore, Watford, Hertfordshire, UK). Standard stock solutions were prepared in methanol at a concentration ranging from 180 to 1000 mg L^{-1} for the five phytoestrogens and kept at −20 °C. The BSTFA:TMCS mixture was prepared under inert gas (N_2) and kept in a glass desiccator to prevent from hydrolyzation of the reagent.

2.2. Instrumentation and GC–MS/MS analysis

The analyses were performed using a Trace GC Ultra gas chromatograph coupled to an ITQ 1100 ion trap mass spectrometer, from Thermo Scientific (Rodano, MI, Italy), equipped with an AI-AS 1310 autosampler. The column used was a Thermo Scientific Trace Gold-SQC 30 m × 0.25 mm ID × 0.25 μm (film thickness), with a composition of 95% methyl polysiloxane and 5% phenyl polysiloxane. The following method was that optimized for trimethylsilyl (TMS) derivatives. The injection was of 1 μL and a programmed temperature vaporizer (PTV) injector was chosen. The injector temperature program was the following: initial temperature of 45 °C was held for 0.35 min during the evaporation phase; temperature increased to 280 °C at 5 °C s^{-1} (held for 1 min) during the transfer phase; in the final cleaning phase temperature increased to 350 °C at 14.5 °C s^{-1} and was held for 10 min with a gas flow of 50 mL min^{-1}, to ensure the elimination of

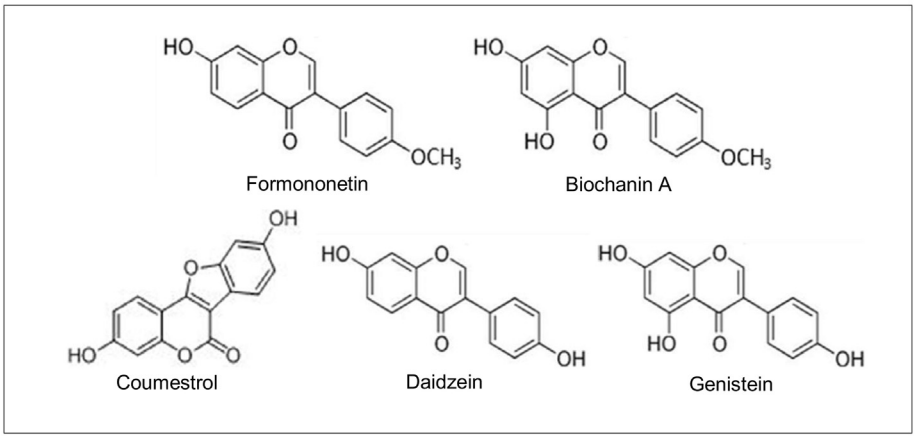

Fig. 1. Structure of the five phytoestrogens studied: formononetin, biochanin A, coumestrol, daidzein and genistein.