PhD Dissertation

DEVELOPMENT AND CRITICAL EVALUATION OF LC-ESI-MS TECHNIQUES BASED ON SPIKING PROCEDURES

DEÁK EDIT

PhD Dissertation

Supervisors:

Éva Stefanovits-Bányai, DSc.

Mihály Dernovics, PhD.

Corvinus University of Budapest

Department of Applied Chemistry

PhD school

Name:	PhD School of Food Science
Field:	Food Science
Head:	**Prof. József Felföldi** CORVINUS UNIVERSITY OF BUDAPEST
Supervisors:	**Éva Stefanovits-Bányai, DSc.** **Mihály Dernovics, PhD.** CORVINUS UNIVERSITY OF BUDAPEST

The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.

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Head of PhD School	**Supervisor**
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	Supervisor

INTRODUCTION

(I) Concentration of ethyl carbamate, a toxic compound that may occur in fermented foods and beverages due to natural biochemical processes has been regulated and limited in several countries such as Germany, USA, Canada, France and the Czech Republic. According to the recent commission recommendation of the European Food Safety Authority (EFSA) issued in March 2010, special attention should be paid to usual and marc spirits distilled from stone fruits as this kind of drinks may contain the highest relative amount of ethyl-carbamate, exceeding sometimes 1 mg l^{-1}. The document highlights also that EU countries should regularly monitor the concentration of ethyl carbamate and report the arising data.

(II) Coenzyme Q_{10} is a key compound involved in the redox cellular metabolism with its primary role in oxidative phosphorylation. It is found both in reduced ($CoQ_{10}H_2$) and oxidized (CoQ_{10}) forms in living cells as the members of the quinone pool localized in the inter-mitochondrial membrane. However it is biosynthesized in humans and it is bioavailable from the diet, there is a general concern about the possible need for supplementation especially for elder individuals with cardiovascular diseases, which is especially supported by its availability from several producers in the EU as registered pharmaceutical drugs intended for chronic cardiac failure. These relationships have triggered a widespread launch of Q_{10} containing dietary supplements in the EU, none the less the EU hasn't included neither forms of Q_{10} in the list published in the Commission Regulation No. 432/2012 about permitted health claims made on foods.Another key issue on Q_{10} containing dietary supplements is whether the oxidized or the reduced form should be encapsulated to provide adequate bioavailability. While most of the supplements contain the more stable CoQ_{10} species, some producers intentionally choose to supply the $CoQ_{10}H_2$ species by formulating the genuine reduced form alone or together with CoQ_{10} to offer a higher positioned product. At the same time, the co-encapsulation of CoQ_{10} in soft gel (liquid) preparations with fat soluble reducing agents such as vitamin E or in hard gel capsules with vitamin C in the presence of emulsifying agents induces the formation of $CoQ_{10}H_2$ during the encapsulation process and storage.

AIMS

The experiments presented in the dissertation can be divided into two main topics as follows:

- To develop a selective and robust HPLCESI-MS/MS method to quantify ethyl carbamate, based on the xanthydrol derivatization technique. The method was validated and applied for a series of authentic traditional and marc spirits.

- To develop a sample preparation technique and an LC-MS method for the quantification of ubiquinol in tablets and hard gel encapsulated dietary supplements. Several extraction techniques including the addition of stabilizing reagents were assessed and a high sample throughput HPLC-ESI-MS/MS method was finally proposed to increase selectivity and to reduce overall labor demand. Also, extraction recovery and total CoQ_{10} were checked with an HPLC-UV method for quality control purposes.

MATERIALS AND METHODS

QTRAP 3200 triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems/Sciex; Foster City, CA, USA) was used in multiple reaction monitoring (MRM) mode. The instrument was equipped with a Turbo V interface and Turbo Ion Spray probe (Applied Biosystems), operating in positive ion mode. The HPLC-ESI-MS coupling was achieved by using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) with a Waters Xterra MS C18 HPLC column (2.1 mm × 20 mm × 5 μm) used with isocratic elution, and for the determination of ethyl-carbamate an Agilent Zorbax XDB-C18 reverse phase column (4.6 mm×150 mm×5 μm) was eluted in gradient mode.

Xanthyl-ethyl carbamate standard solution was prepared according to Moskalyk and Chatten (1967). Briefly, 2.0 g (0.01 mol) xanthydrol and 1.0 g (0.01 mol) ethyl carbamate were mixed in 15.0 ml of glacial acetic acid and heated at 40 °C for 30 min. The product, 9-xanthyl-ethyl-carbamate, crystallized readily and was filtered and washed with 250 ml ice-cold deionized water. The product was recrystallized from 1,4-dioxane:water:acetone (4:4:2) mixture and resulted in white crystals that were washed with ice-cold methanol (25 ml) and air-dried. The yield of the process was 835 mg (28%).

$CoQ_{10}H_2$ standard solution was prepared from CoQ10 according to the method of Yamashita and Yamamoto. Briefly, CoQ10 was reduced and converted to $CoQ_{10}H2$ with $NaBH_4$: 2.0 ml of 25 μg ml^{-1} CoQ_{10} dissolved in hexane was completed 50 μl methanol and 20 mg $NaBH_4$, and the mixture was stirred for three minutes and kept at room temperature in the dark for five minutes. Complete reduction was indicated by the disappearance of the yellow color of CoQ_{10}. Afterwards 1.0 ml of 0.1 M EDTA solution in water was added and the tube was shaken and centrifuged for three minutes at 2000 *g*. Since aqueous methanol is not miscible with hexane, and $CoQ_{10}H_2$ does not partition into the aqueous methanol phase, the concentration of $CoQ_{10}H_2$ in the hexane phase should be the same as the initial CoQ_{10} concentration, in both cases 25 μg ml^{-1}.

RESULTS

The recent promotion of Hungarian „pálinka" brewing and the regulative issues of the EU on the decreasing of ethyl carbamate intake were the leading forces in my study to establish a novel analytical method for the quantification of ethyl carbamate. The approach was based on LC-ESI-MS, the plartform routinely used in food analytical laboratories that could provide a method with a set of analytical parameters exceeding those of the operative official method described in the „Hungarian Book of Wines" for almost all of the attributes. One of the important new features of my method was the ESI-MS characterization of xantil-ethyl-carbamate, the derivative that could make the whole procedure selective and sensitive enough. The final method achieved the accreditation level and it could provide accurate results for LRM samples. The quality of the method was not diminished by the unsuccessful introduction of internal standardization with imazalil and buthyl carbamate as the applied spiking procedure could compensate for analytical losses and drifts.

The ubiquinol content of foods and food supplements has usually been considered as an analytical interference that calls for uniformization during sample preparation. On the other hand, market positioning and involving some recent reports on the physiological attributes of ubiquinol, there has been a growing interest in the quantification of this reduced form of coenyzme Q_{10} – however, analytical methods developed in the human clinical area cannot be directly applied. In my study, the analysis of food supplements containing Q_{10} in a concentration range exceeding human physiological samples with 3-5 orders of magnitude was targeted for ubiquinol quantification. The method development process included (i) the preparation and validation of ubiquinol standard solution, (ii) the assessment of oxidative properties of extraction methodologies, (iii) the AOAC-method based validation of the extraction efficiency and the decomposition free character of the sample preparation, and (iv) the establishment of a novel spikig method to provide accurate results. Finally, half of the analysed food supplement samples contained ubiquinol. As no indication about this compound was given on any labels of the products tested, the formation of ubiquinol may have originated from unintentional reduction of the oxidised form of Q_{10} during either the production or the packaging processes. The extended uncertainty budget of the procedure concluded that the step responsible for the highest contribution was the standard deviation of the ESI-MS instrument. Indeed, 1% of standard deviation of the ESI-MS resulted in +/- 25% uncertainty of the final analytical result.

LIST OF PUBLICATIONS

International conference abstracts:

1. E. Deák, A. Gyepes, M. Dernovics, É. Stefanovits-Bányai
Determination of ethyl-carbamate for authentification of Hungarian cider spirits by HPLC-ESI-MS
Recent Advances In Food Analysis (RAFA), 4-6/11/2009, Prague, Czech Republic

2. E. Deák, A. Gyepes, M. Dernovics, É. Stefanovits-Bányai
Derivatization based enhanced selectivity of ethyl-carbamate determination in spirits by HPLC-ESI-MS/MS hyphenation
7th Aegean Analytical Chemistry Days, 29/09-04/10/2010, Lesbos, Greece

Peer reviewed journal articles:

1. Edit Deák, Attila Gyepes, Éva Stefanovits-Bányai, Mihály Dernovics
Determination of ethyl carbamate in pálinka spirits by liquid chromatography–electrospray tandem mass spectrometry after derivatization
Food Research International, 43 (2010) 2452–2455
IF (2010): 2.416
Number of independent citations according to Scopus, accessed at 03/11/2014: 17

2. Andrea Vass, Edit Deák, Mihály Dernovics
Quantification of the reduced form of coenzyme Q_{10}, ubiquinol, in dietary supplements with HPLC-ESI-MS/MS
Food Analitical Methods, 2014, DOI 10.1007/s12161-014-9911-x
IF (2013): 1.802