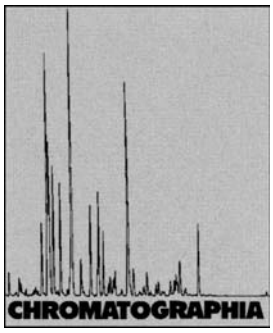
Modification of Chromatographic Method for the Determination of Benzoquinones in Cereal Products



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Abstract

The 2,6-dimethoxy-p-benzoquinone (2,6DMBQ) derived from different plant species is of special interest. The wheat (Triticum vulgaris) is one of the largest known natural sources of these compounds. These compounds occur in glucoside form, and are located in the embryo. In the present work we aimed at developing chromatographic method for precise, sensitive and reliable analysis with high stability of benzoquinones. In the newly worked out method RP-amide C16 column was used with 20% acetonitrile in phosphate buffer as the mobile phase. The method was validated according to calibration curve, precision and recovery tests, limit of detection and quantitation. The results indicated that as low as 0.27 μg/mL can be quantitatively determined by this method with 100.5–101%, and 97% for recovery and precision, respectively. There was significant reduction in the time required for the complete elution of phenol compounds with substantial improvement on the symmetry of their peaks as compared to those noticed with other methods. The standard and sample solutions showed higher stability at refrigeration storage for more than 4 months. As an application of the new method, fermented wheat germ, germinated wheat and extract of wheat seedlings were analysed for their benzoquinone content. The highest level was found in the fermented wheat germ, while minute quantities could be detected in the other samples.

Keywords

Column liquid chromatography
Benzoquinones
Content in cereals and wheat

Introduction

Naphto-, hydro- and benzoquinones are of the naturally occurring quinones that have very interesting biological role. Most of them function as antioxidants in biological matrices. In specific works some benzoquinones such as 2,6-DMBQ have been proved to exhibit cytotoxic effect in the Ehrlich Ascites Tumor Cells (EATC) [1–4], and thereby inhibit tumor propagation and metastases. Recent studies [5–7] showed a benzoquinone component 2,6-DMBQ from fermented wheat germ product (Avemar) induces apoptosis and down regulation of the MHC (Main Histocompatibility) Class I antigenes in the tumor T and B cell lines. The low level of these antigens enhances the activity of NK-cells (Natural Killer), which play important role in the immunresponse against cancer.

Benzoquinones from biological materials have been determined by paper- and thin-layer chromatography [8], combination of gel permeation and liquid chromatography [9], high performance liquid chromatography [10], micellar electrokinetic chromatography [11] and most recently extensive spectroscopic analysis using IR, NMR, MS, HRMS as well as HPLC and GC-MS [12].

In the HPLC methods elaborated, so far, to separate substituted p-benzoquinones and p-hydroquinones reversed phase C18-columns have been used with combinations of methanol and either water [9, 10] or phosphate buffer [13] as the mobile phase. These methods when applied under the conditions of our laboratory had some limitations concerning either retention reliability, peak broadening, re-equilibration of column, sensitivity or stability of material in standard and sample solutions.

The objective of the present work was to elaborate sensitive, reliable HPLC method, elongate lifetime of column and increase storage stability of working and stock solutions of benzoquinones.

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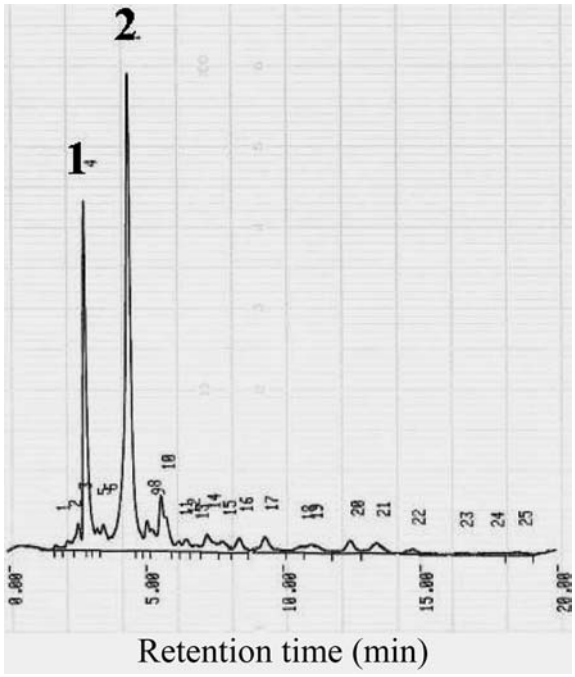

Fig. 1. HPLC chromatogram of benzoquinones from wheat germ. Fore conditions see text. 1: 2-MBQ, 2: 2,6-DMBQ

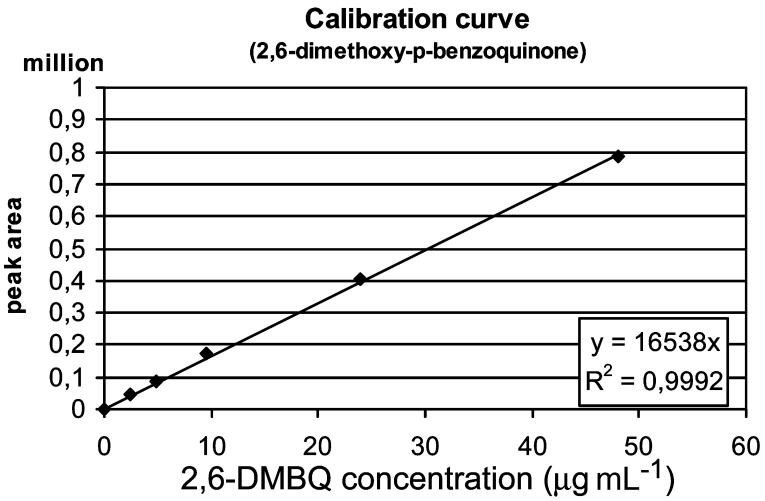

Fig. 2. Calibration curve of 2,6-DMBQ as determined by the newly elaborated HPLC method

Table 1. parameters used for validation of the newly elaborated HPLC method

Parameters	Previous method	New method
LOD	0.5 µg mL^{-1}	0.08 µg mL^{-1}
LOQ	1.7 µg mL^{-1}	0.27 µg mL^{-1}
Relative Standard Deviation	5–10%	3–3.5%
Measurement time	50 min	20 min
Sample weight	2 g	0.5 g
Regeneration of column	After 10 injections	After 100 injections

Experimental

Materials

The standard 2,6-DMBQ (2,6-dimethoxy-*p*-hydroquinone) and 2-MBQ (2-methoxy benzoquinone) were synthesized in the Department of Organic Chemistry, Budapest University of Technology and Economics. The other chemicals, such as anhydrous Na_2SO_4, KH_2PO_4, acetonitrile, chloroform were purchased from REANAL Fine Chemicals Co.(Budapest, Hungary). All reagents were used without additional cleaning. Wheat and barely seeds were obtained from the Research Institute of Cereals (Szeged, Hungary)

Methods

Sample Preparation

Two grams of food sample were dissolved in 50 mL of doubly distilled water and then extracted three times by shaking with 25 mL of chloroform. The chloroform layers were pooled, washed twice with distilled water and dried over anhydrous Na_2SO_4. The filtrate was evaporated to dryness by vacuum evaporator at maximum 40 °C. Dry material was finally redissolved in 5 mL of eluent and pre-injection filtration with PTFE 0.45 µm filter aid was done. Twenty µL of the final filtrate were injected into the HPLC column.

Instruments and Chromatographic Conditions

The chromatograph consisted of a Beckman114M pump, a Beckman System Gold Model 166 UV-VIS detector and Waters 740 type integrator. Discovery Rp-amide C16 , 5 µm 250 × 4.6 mm Column was used with 20% acetonitrile in 0.025M KH_2PO_4, pH = 4.8 as the mobile phase. The isocratic elution was performed at flow rate of 0.7 mL min^{-1}. The column effluent was monitored at 290 nm.

Result and Discussion

In the first attempt to determine 2,6-DMBQ in wheat products the method modified by Huang et al. [13] was adapted. There was no problem with the separation of 2,6-DMBQ and 2-MBQ. However, the column needed frequent washing and re-equilibration to get somewhat reproducible analysis. Furthermore stability of standard solutions and sample extracts was low when prepared with methanol or HPLC eluent containing methanol and ammonium hydroxide and Na-EDTA. With acetonitrile containing phosphate buffer, benzoquinones were highly stabile for 1 month at refrigeration temperature. The marked decrease in the concentration of these compounds was observed after a 3-month storage.

Table 2. Recovers of 2,6- DMBQ after spiking benzoquinone free wheat sample with known quantities of standard material

Sample	Quantities	Recoveries %
1	0	0
2	1.5 mg standard	101
3	0.012 mg standard	100.56

Table 3. Content of 2,6-DMBQ in some foods as estimated by the newly elaborated HPLC method

Food	Concentration of 2,6-DMBQ [mg g^{-1}]
Wheat germ	0.1
Fermented wheat germ extract	0.2
Germinated wheat product	0.001
Liquor of germinated wheat	0.002 [mg mL^{-1}]
Barley germ	0.07

Method Development and Validation

Since benzoquinones are compounds of relatively low hydrophobicity, a modified reversed-phase sorbent was needed to provide better resolution with a mobile phase containing high level of organic modifier such as acetonitrile, in which benzoquinones are easily soluble and highly stable.

The separation was performed on RP-C18 columns (Kromsil) with different pore-size (100–120 Δ) using mobile phases containing acetonitrile up to 30% as modifier in attempt to resolve different benzoquinone-derivatives. However, none of them was successful. The best separation could be achieved on RP-amide C16 (Discovery from Supelco) using mobile phase consisting of 20:80 acetonitrile-phosphate buffer at pH 4.8. Resolution of 2-MBQ and 2,6-DMBQ was >1.5 and the greatest asymmetry value was <2.0.

Because of its being the most important effective material in germinated wheat products the work focused only on 2,6-DMBQ. With 150×4.6 mm column there was some overlap between the 2-MBQ and 2,6-DMBQ, but on 250×4.6 column of the same sorbent the two benzoquinones could be well separated. The amide-C16 column gave possibility to apply the modifier acetonitrile at a wide range between 5% and 40% with high peak symmetry and sensitivity for benzoquinones and degraded components (Fig. 1).

Linearity

Each of the five solutions of different concentrations for 2,6-DMBQ was injected several times onto the HPLC column. The average peak area was plotted versus concentration (Fig. 2). A linear response between peak area and concentration was observed. The correlation coefficient (r^2) was 0.9992.

Detection Limits and Precision

As shown in Table 1 limit of detection (LOD) and limit of quantitation (LOQ) for 2,6-DMBQ were 0.08 and 0.27 μg mL^{-1} respectively. These values are well above the 0.05 and 1.7 μg mL^{-1} obtained with the HPLC method using 20% of methanol in $(NH_2OH)PO_4$–NaEDTA as the mobile phase on RP-C18 columns.

Precision of method expressed as relative standard deviation was also improved. It could be increased to 3,5–4%. Since the extraction and sample preparation was the same in the two methods the increased precision is most probably due to minimizing of on-column degradation of benzoquinones, which have been mentioned to be the most serious problem in the HPLC determination of such components.

The increased sensitivity of the HLPC method assisted to reduce sample weight from 2–0.5 g and shorten the time required for complete elution of MBQ-s and accompanying materials (unidentified) to be 20 min instead of 50 min with the other method.

As a result of use of acetonitrile up to 20–30% washing and re-equilibration of column was needed after move than 100 injections. This well compares to the frequent re-equilibration (after 10 injections) required with the other methods.

Recoveries

Recovery of 2,6-DMBQ was checked by spiking wheat sample (free of this compound) with known quantities from standard material. The same extraction and HPLC determination procedures were used as in the analysis of food samples. The results (Table 2) showed that recovery of 101% and 100,6% could be achieved when the amounts of 1.5 mg and 0.012 mg were spiked respectively.

2,6-DMBQ in Food

It is well known that the germs of some cereal crops such as wheat, triticale and barley are the mean source of benzoquinones in human diets. Therefore, some foods where benzoquinone are expected to occur were analyzed. Table 3 summarizes the obtained results from HPLC determination of 2,6-DMBQ in wheat and barley product. The highest level of this compound was found in the fermented product of wheat germ, which contained 0.2 mg g^{-1} while the unfermented wheat germ contained about 0.1 mg g^{-1}. This revealed the release of bound 2,6-DMBQ by some hydrolyzing enzymes during fermentation process. In germinated wheat products and wheat liquor minute quantities (0.001–0.002 mg g^{-1}) were found indicating that germination of wheat seed reduces the level of 2,6-DMBQ. The 0.07 mg g^{-1} found in barley germ is well below the values estimated for wheat germ product. This means that the germs of different cereal crops vary significantly in the content of biologically important benzoquinones.

Acknowledgement

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