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ORIGINAL ARTICLE

Fast analysis of wine for total homocysteine content by high-performance liquid chromatography

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Abstract Alimentary methionine is believed to be the main source for plasma homocysteine. Recent literature supplies information about homocysteine content in daily food components, but not in wine, an attractive complement of the evening meal in some western countries. In this communication, a simple and fast high-performance liquid chromatography method for determination of total homocysteine in wine is described. The two steps procedure relies on reduction of the disulfide forms of homocysteine with tris-(2-carboxyethyl)phosphine and on-column derivatization with *o*-phthaldialdehyde followed by separation and fluorescence detection. The entire analysis time, including sample work-up, amounts 14 min. The calibration performed with wine matrix, spiked with homocystine within the practical concentration range, proved linear response of the detector. The proposed method was applied for the analysis of 32 different types of wines for total homocysteine. The average concentration of the analyte was 10.31 (±4.25) μM and 6.11 (±3.44) μM for red ($n = 23$) and white ($n = 9$) wines, respectively.

Keywords Homocysteine · Wine · Determination · Liquid chromatography

Abbreviations

Hcy	Homocysteine.
tHcy	Total homocysteine
$(Hcy)_2$	Homocystine
TCEP	*tris*-(2-Carboxyethyl)phosphine
OPA	*o*-Phthaldialdehyde
LOQ	Limit of quantitation
LOD	Limit of detection
RSD	Relative standard deviation

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Introduction

Homocysteine (Hcy) is an endogenous sulfhydryl amino acid generated by the demethylation of methionine which is present in food especially in meat. Once formed, homocysteine is either catabolized by trans-sulfuration to cysteine or remethylated to methionine (Ducros et al. 2002). In healthy humans, total plasma Hcy level is regulated and the normal basal concentration ranks from 5 to 15 μM, with a mean level of about 10 μM (Refsum et al. 1998). The state of organism, with total plasma homocysteine level greater than 15 μM, is termed hyperhomocysteinemia. Statistically, mild hyperhomocysteinemia (total plasma Hcy concentration within 16–30 μM) concerns about 7% of general population of high developed countries (Jacobsen 1993). From several years, high Hcy level is considered to be an independent risk factor for cardiovascular diseases, including coronary artery disease, cerebrovascular diseases and peripheral vascular diseases. An elevated plasma Hcy concentration may occur as the result of some diseases, medications, unhealthy lifestyle or nutritional deficiencies of the vitamin cofactors (Fonseca et al. 1999; Refsum et al. 1998; Eikelboom et al. 1999). In the case of moderate alcohol consumption, the reported results are inconsistent. Several reports suggest that

moderate alcohol consumption, but not beer (Sakuta and Suzuki 2005), positively correlates with plasma total Hcy (Bleich et al. 2001; Jacques et al. 2001), but on the other hand quite a few of reports show that intake of beer or wine decreased (Burger et al. 2004; De Bree et al. 2001; Ubbink et al. 1998), or unchanged (Ayaori et al. 2000) total concentration of this amino acid. Probably, the most discussed issue is the so-called "French paradox". The 1970s cardiovascular diseases prevention message coming from USA was discouraging. It dictated avoidance of cigarettes, significant amount of alcohol, coffee and fatty and rich foods. The French are not compatible with the conditions. They used to enjoy rich food with saturated fat and wine, similar cholesterol and homocysteine levels to other nations, and a very low coronary heart disease mortality (Rajdl et al. 2007). The apparent discrepancy of the findings may partly be ascribed to the differences in the types of alcoholic beverages consumed. However, one of the most recent reports states that changes in plasma Hcy concentration are not associated with total alcohol or wine consumption (Husemoen et al. 2009). Hcy is formed from methionine, but also is provided with food. There is no study addressing the question whether dietary Hcy taken up with food may contribute to total plasma Hcy, although Hcy has been quantified in a variety of animal tissues and plants (Demirkol et al. 2004). There are only a few reports describing content of Hcy in food and beverages, among others in beer, but not in wine (Pexa et al. 2008; Sakamoto et al. 2002).

In this paper, we describe a simple and sensitive HPLC method for determination of tHcy based on on-column derivatization with *o*-phthaldialdehyde (OPA) and fluorescence detection. The method was applied for the analysis of 32 red and white wine samples of different origin. To the best our knowledge, homocysteine was not determined in wines so far.

Materials and methods

Chemicals and reagents

D, L-homocystine $(Hcy)_2$, D, L-homocysteine (Hcy) were from Sigma (St. Louis, MO, USA). Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) was obtained from Sigma (St. Louis, MO, USA). *o*-Phthaldialdehyde (OPA) was from Fluka (Buchs, Switzerland). Sodium hydroxide (NaOH) was obtained from Merck (Darmstadt, Germany). LC-grade acetonitrile was from Labscan (Dublin, Ireland). Sodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) were received from J.T. Baker (Deventer, The Netherlands).

Instrumentations

Chromatographic analyses were performed on a Hewlett-Packard 1100 Series (Waldbronn, Germany) system encompassing quaternary pump, autosampler, thermostated column compartment, vacuum degasser, 1046A fluorescence detector, and controlled by HP ChemStation software. For pH measurement, a Hach One 43 800–00 pH-meter (Loveland, USA) was used.

Collection of samples

Wine in 0.7 L glass bottles imported from different countries was purchased in local supermarkets.

Sample preparation

For the determination of total homocysteine (tHcy) to 50 μL of wine, 190 μL of 0.2 M (pH 7.4) phosphate buffer, and 10 μL 0.25 M TCEP were added. The mixture was incubated for 10 min at room temperature and 10 μL of the final analytical solution was injected into the chromatographic column.

On-column derivatization and separation

The final analytical solution (10 μL) was injected using an autosampler into a PRP-1 Hamilton C-18 (150 × 4.6 mm) column packed with 5 μm particles (Energy Way, Reno, Nevada, USA) and eluted isocratically. The mobile phase consisted of a mixture 0.01 M OPA in sodium hydroxide (0.1 M) solution and acetonitrile in the ratio of 7:3. The temperature was 25°C and the flow rate 1 mL/min. The excitation and emission wavelengths of the fluorescence detector were fixed at 370 and 480 nm, respectively, and gain 14. Identification of peaks was based on the comparison of retention time with corresponding set of data obtained for authentic compound.

Calibration

Stock solution of $(Hcy)_2$ (10 mM) for method development and validation procedure was prepared by dissolving appropriate amount of the compound in 0.01 M HCl. Working standard solutions were prepared by dilution with water when required. To prepare calibration standards, 50 μL of wine samples was each placed in polypropylene tube and spiked with the growing amount of working standard solution of the analyte at seven levels of concentration. The calibration range was 0.1–16 (0.1, 0.5, 1, 2, 4, 8, 16) μM tHcy in wine. Calibration standards were subjected to recommended procedure for tHcy measurement. The calibration curve was obtained plotting the peak

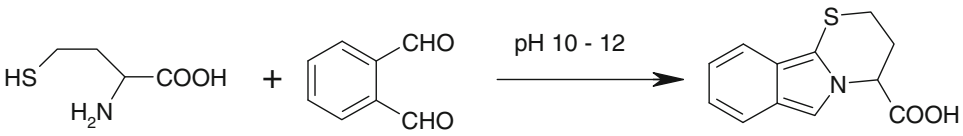

Fig. 1 Derivatization reaction equation of homocysteine with *o*-phthaldialdehyde

heights against the thiol concentrations. The limit of detection (LOD) was calculated as signal to noise ratio of 3, and the lower limit of quantification (LOQ) was the lowest concentration on the standard curve that can be measured with acceptable accuracy and precision.

Results and discussion

In this report we present a modification of an HPLC-OPA method, described recently for determination of protein N-linked homocysteine, homocysteine and homocysteine thiolactone in plasma and urine (Głowacki et al. 2010a; Głowacki et al. 2010b), to determination of total homocysteine in wine. First step of the recommended procedure consists of reduction since the bulk of Hcy in wine, similar to other biological samples, occurs in the disulfide form not accessible for the derivatization reagent. Here, for this purpose TCEP solution was used. The second step constitutes on-column derivatization and separation.

Derivatization and separation

Under the conditions described in Experimental section Hcy reacts instantaneously on-column with OPA, present in the mobile phase, to give an OPA-Hcy derivative according to the equation shown in Fig. 1. The continuous variation method has shown that substrates react in the molar ratio of 1:1 (Fig. 2). The derivative eluted under isocratic conditions after 2.1 (±0.016, $n = 20$) min as depicted in Fig. 3.

Method validation

Method validation protocol encompassed linearity, imprecision, recovery, LOD and limit of quantification (LOQ). A linear relation was obtained between peak height and Hcy concentration in the studied range. The equation for the regression line was $y = 2.45x + 21.04$. Standard errors for slope and intercept were 0.018 and 0.943 %F/mol × L, respectively. Regression analysis of the linearity has shown correlation coefficient of $R = 0.9999$. Recovery and imprecision were studied for three concentrations, representing whole range of the calibration curve: one near the LOQ, one near the center and one near the upper boundary of the standard curve. The mean imprecision (RSD% $n = 5$) and mean recovery (%) were 1.45 and 99.5,

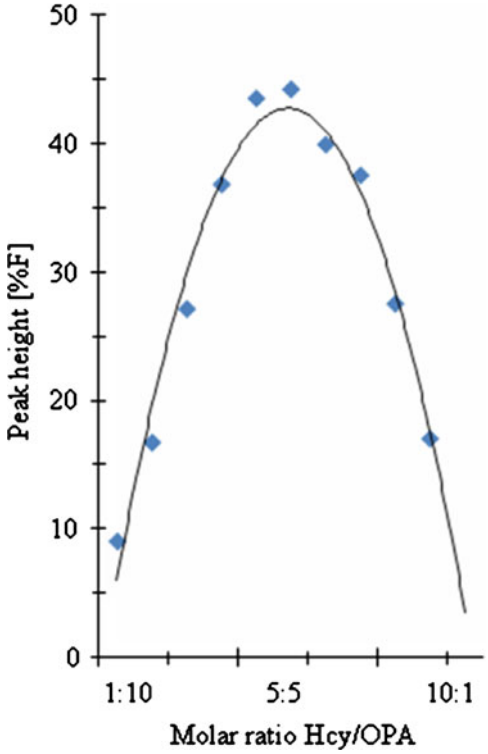

Fig. 2 Estimation of stoichiometric molar ratio by continuous variation method for the reaction of homocysteine with *o*-phthaldialdehyde

respectively. The LOD and LOQ of the assay were 0.05 and 0.1 µM, respectively.

Application of analysis to real samples

The proposed method was applied for the analysis of 32 different samples of wine, imported from several countries, for total homocysteine. As shown in Table 1, the content of tHcy in a variety wines differs significantly. In general, red wines are richer in Hcy than white ones, 10.31 (±4.25, $n = 23$) versus 6.11 (±3.44, $n = 9$) µM. Regardless the color Spanish wines showed almost threefold higher Hcy content (17.65 ± 5.7) when compared with California wines (6.67 ± 1.98). Because none of Hcy was found in grapes (Demirkol et al. 2004, and our unpublished results) or as little as 0.01 mg/100 g grapes (Sakamoto et al. 2002) we conclude that this sulfur amino acid is formed during fermentation process from sulfur substrates. Therefore, the level of Hcy in wine may depend on various conditions

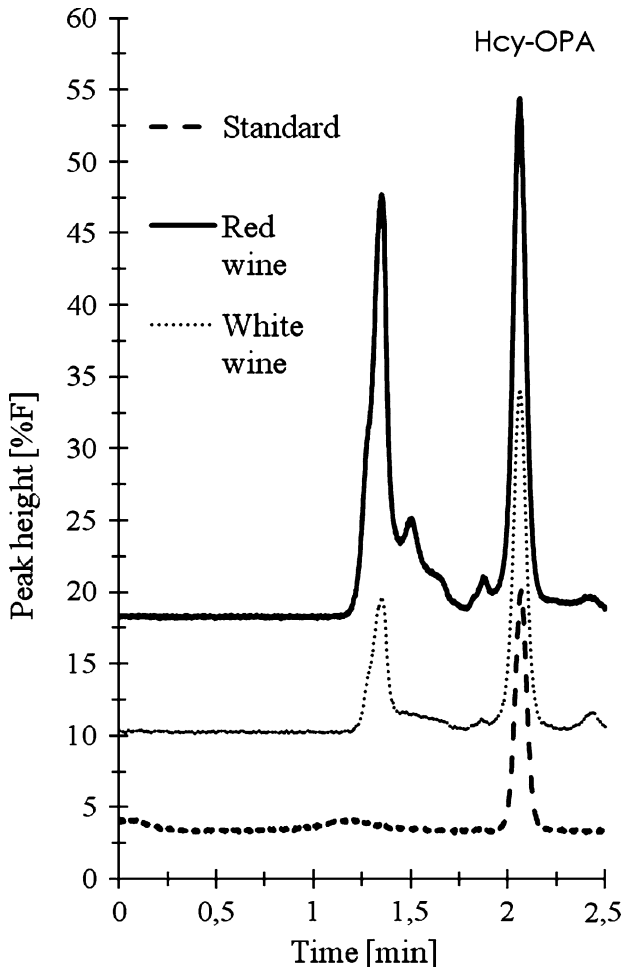

Fig. 3 HPLC analyses of standard water solution and wine for homocysteine using on-column derivatization with *o*-phthaldialdehyde and fluorescence detection. Capacity of peaks: water standard solution, 8 μM; white wine, 9.8 μM; and red wine, 12.3 μM Chromatographic conditions as described in text

such as climate, species of vine, region where the grapes are harvested, and yeast used for fermentation.

To the best of our knowledge, there is no study addressing the question whether dietary Hcy taken up with foods, including wine, may contribute to plasma homocysteine. When considering plasma Hcy levels of 5–15 μM, we believe that the amount of Hcy introduced with wine has only a very slight impact on its plasma content even if complete resorption of the thiol is assumed.

Table 1 Analysis of wines for total homocysteine

Number of sample	tHcy (μM)	SD (μM)	RSD (%)	Country of origin
White wine				
1	0.96	0.05	4.7	Moldavia
2	1.81	0.03	1.63	Bulgaria
3	4.50	0.00	0.00	USA (California)
4	5.13	0.06	1.15	Portugal
5	5.79	0.06	1.02	USA (California)
6	7.84	0.06	0.75	USA (California)
7	8.02	0.27	3.31	USA (California)
8	9.77	0.15	1.51	Bulgaria
9	11.19	0.50	4.48	Czech Republic
Red wine				
1	4.17	0.20	4.80	Italy
2	4.44	0.09	1.99	Bulgaria
3	4.63	0.18	3.91	USA (California)
4	7.27	0.21	2.84	France
5	7.27	0.09	1.22	Bulgaria
6	7.52	0.32	4.31	France
7	8.46	0.38	4.57	Argentina
8	8.57	0.15	1.72	France
9	8.92	0.12	1.32	Poland
10	9.27	0.03	0.32	USA (California)
11	9.92	0.49	4.94	Romania
12	10.05	0.24	2.35	Hungary
13	10.36	0.32	3.13	Hungary
14	10.46	0.29	2.82	Chile
15	10.96	0.29	2.69	Bulgaria
16	10.96	0.29	2.69	Argentina
17	11.32	0.38	3.39	Bulgaria
18	12.34	0.41	3.34	Argentina
19	12.50	0.29	2.36	Chile
20	14.07	0.15	1.05	Spain
21	14.63	0.53	3.63	Spain
22	14.78	0.44	2.99	Bulgaria
23	24.24	0.15	0.61	Spain

Conclusions

In conclusion, presented method easily and reliably measures total homocysteine in wine within 14 min including sample work-up. The analytical figures of merit, linearity, precision and recovery, shown during the method validation procedure, are well within the criteria for biological sample analysis (FDA Guidance for Industry Bioanalytical Method Validation 2001). To our best knowledge, a method for determination of Hcy in wine has not been described in the literature so far, and proposed in this report assay, compares well with methods for homocysteine, potentially capable to measure this amino acid in wine (Bald and Głowacki 2005; Sakamoto et al. 2002; Pexa et al.

2008) in terms of overall analysis time and quality of HPLC separation. This new analytical procedure should contribute to the elucidation of the influence of alimentary homocysteine on its plasma content.

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