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Reduced and total glutathione and cysteine profiles of citrus fruit juices using liquid chromatography

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Abstract

A liquid chromatography assay for the determination of different species of glutathione and cysteine in fruit juices is described. The method is based on derivatization of thiols with 2-chloro-1-methylquinolinium tetrafluoroborate followed by chromatographic separation and UV-absorbance detection and quantitation. The method is linear in wide range of concentrations with a regression coefficient better than 0.99. The detection limits for glutathione and cysteine were 0.1 and 0.05 μmol L^{-1}, respectively. Analytical recovery and the imprecision for both analytes were in the ranges 99.1–101.3% and 2.0–9.0%, respectively. The method was successfully applied to analysis of orange and grapefruit juices for reduced and total glutathione and cysteine.

Keywords: Glutathione; Cysteine; Fruit juices; Liquid chromatography

1. Introduction

Low molecular-mass thiols and their disulfides are critical cellular components that play numerous important roles in metabolism and in the antioxidant defense network. The main thiols in plants are glutathione (GSH) and cysteine (CSH), which are involved in various aspects of plants physiology, including electron transport and phosphorylation during photosynthesis (Saetre & Rabenstein, 1978). Biomedical researchers realize that glutathione is a major antioxidant and detoxifier with many essential metabolic functions in humans (Meister, 1989). Enhanced tissue levels resulting from dietary glutathione and cysteine, its main amino acid precursor, suggest their role as nutrients. Glutathione and cysteine are present in most plant and animal tissues from which the human diet is derived. It has been well established that dietary GSH enhances metabolic clearance and decreases net absorption of dietary peroxidized lipids (Kowalski, Feeley, & Jones, 1990; Yee & Williams, 1992). Moreover, Flagg et al. (1994) reported that consumption of food high in glutathione reduce significantly the risk of oral and pharyngeal cancer. Both thiols were determined in food, including fruits and vegetables (Demirkol, Adams, & Ercal, 2004; Jones, 1995; Jones et al., 1992; Mills, Stinton, Liu, & Lang, 1997; Saetre & Rabenstein, 1978), but there is relatively little quantitative information about the levels of their disulfide forms.

The purpose of this study was to develop a liquid chromatography method for the determination of different species of glutathione and cysteine in fruit juices. The method relies on transformation of thiols, in the reaction with ultraviolet thiol-specific tagging reagent – 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) (Bald & Głowacki, 2001) to stable derivative, and separation and quantitation by ion-pairing reversed-phase liquid chromatography. For total thiol content, oxidized species are converted by reduction to the thiol form before the derivatization step. The difference between total content of a thiol and content of its reduced form constitutes disulfide form.

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2. Material and methods

2.1. Chemicals, reagents and apparatus

2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) was prepared in this laboratory according to the procedure described earlier (Bald & Głowacki, 2001). Perchloric acid (PCA), hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) and HPLC-grade acetonitrile were from J.T. Baker (Deventer, Netherlands). Trichloroacetic acid (TCA), sodium borohydride ($NaBH_4$) and lithium hydroxide monohydrate ($LiOH \cdot H_2O$) were from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) and *n*-octanol were obtained from Pierce (Rochford, USA). L-Cysteine (CSH), L-cystine (CSSC) and reduced (GSH) and oxidized (GSSG) glutathione were obtained from Reanal (Budapest, Hungary).

Stock standard solutions of calibrators of thiols and their disulfides (0.1 mol L^{-1}) were prepared by dissolving an appropriate amounts of the compounds in 0.1 mol L^{-1} HCl. Solution of $NaBH_4$ (6 mol L^{-1}) was prepared by dissolving appropriate amount of the compound in 0.1 mol L^{-1} sodium hydroxide and diluted 2:1 with dimethyl sulfoxide. For derivatization a 0.1 mol L^{-1} water solution of 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) was used. To prepare phosphate buffer (0.2 mol L^{-1}) appropriate quantities of sodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) and sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) solutions were mixed. TCA buffer was prepared using 0.05 mol L^{-1} trichloroacetic acid and adjusted to the pH 3.0 with lithium hydroxide monohydrate at the same concentration. The pH of the buffers was adjusted by potentiometric titrations. All the stock solutions were stored in the refrigerator at +4 °C.

HPLC analyses were performed with a Hewlett-Packard 1100 Series system (Waldbronn, Germany) equipped with quaternary pump, an autosampler, thermostat, vacuum degasser and DAD-UV detector. For instrument control, data acquisition and data analysis a HP ChemStation for LC 3D system including single instrument HP ChemStation software and Vectra color computer was used. The separations were accomplished with a Zorbax SB C-18 (5 μm, 150 × 4.6 mm) analytical column (Agilent Technologies, Waldbronn, Germany). For pH measurement a HI 221 (Hanna Instruments, Woonsocket, USA) pH meter was used. Water was purified using a MILLI-Q-RG system (Millipore, Vienna, Austria).

2.2. Analytical procedure

For the determination of reduced thiols to 150 μL of fruit juice, 200 μL of 0.2 mol L^{-1} pH 7.6 phosphate buffer and 20 μL of 0.1 mol L^{-1} CMQT were added. After 1 min 100 μL of 3 mol L^{-1} PCA was added and the mixture was centrifuged (5 min, 12,000*g*). Next, sample was transferred into the autosamples vial, followed by injection (20 μL) into the chromatographic system.

For the determination of total thiols (assumed as sum of reduced and oxidized forms) to 150 μL of fruit juice, 50 μL of *n*-octanol, 100 μL of 6 mol L^{-1} $NaBH_4$ in 0.1 mol L^{-1} NaOH diluted 2:1 with dimethyl sulfoxide, and 50 μL of 3 mol L^{-1} HCl were added. The mixture was vortex-mixed followed by addition, after 1.5 min, 60 μL of 3 mol L^{-1} HCl in order to decompose excess of sodium borohydride. Next, 100 μL of 0.2 mol L^{-1} pH 7.6 phosphate buffer and 20 μL of 0.1 mol L^{-1} 2-chloro-1-methylquinolinium tetrafluoroborate were added. The mixture was vortex-mixed and put aside for 1 min, acidified with 100 μL of 3 mol L^{-1} perchloric acid followed by centrifugation (5 min, 12,000*g*). A 20 μL of final analytical solution was injected into the chromatographic column.

2.3. Chromatography

Samples (20 μL) were injected using an autosampler into a Zorbax SB C-18 (150 mm × 4.6 mm) column packed with 5 μm particles. The temperature was 25 °C, the flow-rate 1 mL min^{-1} and the detector wavelength was 348 nm. For determination of 2-*S*-quinolinium derivatives of glutathione and cysteine gradient elution was used. The elution profile was as follows: 0–4 min 12% B, 4–7 min 12–25% B, 7–8 min 25–40% B, 8–12 min 40–12% B; where A – 0.05 TCA buffer (pH 3.0) and B – acetonitrile. Identification of peaks was based on comparison of retention times and diode-array spectra, taken at real time of analysis, with corresponding set of data obtained for authentic compounds.

2.4. Calibration

For preparation of calibration standards for determination of reduced and total cysteine and glutathione in fruit juice, portions of 150 μL of juice were each placed in a sample tube and spiked with increasing amount of working standard solutions of CSH, CSSC, GSH and GSSG at six levels of concentration. The calibration ranges were: for reduced cysteine 0.5–10 (0.5, 1, 2, 4, 6, 10) μmol L^{-1}, for total cysteine 1–40 (1, 5, 10, 20, 30, 40) μmol L^{-1}, for reduced glutathione 5–70 (5, 10, 15, 20, 40, 70) μmol L^{-1} and for total glutathione 10–160 (10, 20, 40, 80, 120, 160) μmol L^{-1}. Calibration standards were subjected to all steps of the recommended analytical procedure. The calibration curve was obtained by plotting peak height against the thiol concentration. Lower limits of detection (LLD) were experimentally estimated by analysis of water spiked with decreasing concentrations of the standard analytes until the signal-to-noise ratio of 3:1.

3. Results and discussion

3.1. Reduction and derivatization

Since thiols are present in fruit juice as a free thiol and in various oxidized forms (symmetrical and mixed disulfides) the determination of total amounts must account for all those forms. For this purpose, a reductive cleavage of the disulfides is made before the derivatization and instrumental analysis steps. For reduction, sodium borohydride ($NaBH_4$) was used. During the reduction step disulfides are converted into their thiol derivatives with –SH function accessible to thiol-specific derivatization reagents. For thiol derivatization 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) (Bald & Głowacki, 2001) was used. CMQT reacts rapidly and specifically with the –SH group in slightly alkaline aqueous solution (Fig. 1). The reaction products 2-*S*-quinolinium derivatives, stable thioethers, have a well defined absorption maximum in the higher UV region with a high molar absorptivity coefficient. Bathochromic shift from reagent maximum (328 nm) to the maximum of the derivative (348 nm) is analytically advantageous (Fig. 2). It was thanks to this phenomenon that we could recommend the use of large excess of CMQT in order to drive the reaction to completion (in real world sample) and avoid a huge peak of unreacted derivatization reagent on the chromatogram.

3.2. Chromatogram

The chromatogram of the orange juice after reduction with sodium borohydride and derivatization with 2-chloro-1-methylquinolinium tetrafluoroborate is shown in Fig. 3. At used chromatographic conditions, eluted peaks were distinctly separated. Unidentified peaks appeared but did not interfere with the peaks of interest. 2-*S*-quinolinium derivatives of glutathione and cysteine were eluted after 3.35 (RSD 0.20%, $n = 21$) and 9.00 (RSD 0.15%, $n = 21$) min, respectively.

3.3. Validation study

The linearity of relationships between thiol concentration and peak height of the respective analyte-CMQT derivative was determined by analysis of fruit juice spiked with standard solutions of the thiols and processed according to the recommended procedure. Seven-point calibration plot was constructed using triplicate injections of the final analytical solution. Outliers were not excluded. The calibration curves for reduced and total glutathione and cysteine were linear in the tested ranges with correlation coefficients (R^2) better than 0.99 for all analytes. The equations for the regression line ($n = 3$) were: $y = 1.2371x + 26.881$ for reduced glutathione, $y = 0.8522x + 43.316$ for total glutathione, $y = 2.6988x + 8.2837$ for reduced cysteine and $y = 2.203x + 16.179$ for total cysteine (where y is the peak height and x is the concentration of the analyte). The lower limits of determination of glutathione and cysteine were 0.1 and 0.05 μmol L^{-1}, respectively. Percentages of recoveries and between analyses imprecision,

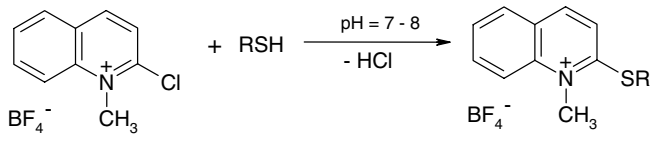

Fig. 1. Derivatization reaction equation of thiol with 2-chloro-1-methylquinolinium tetrafluoroborate.

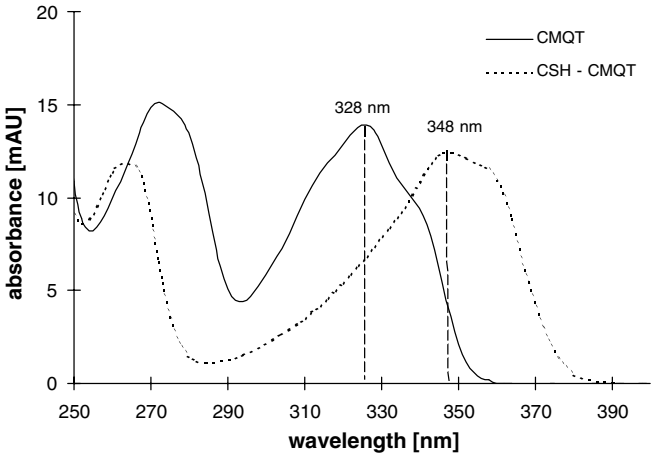

Fig. 2. Comparison of the absorption spectra of derivatization reagent – CMQT (continuous line) and cysteine derivative – CSH-CMQT (dotted line).

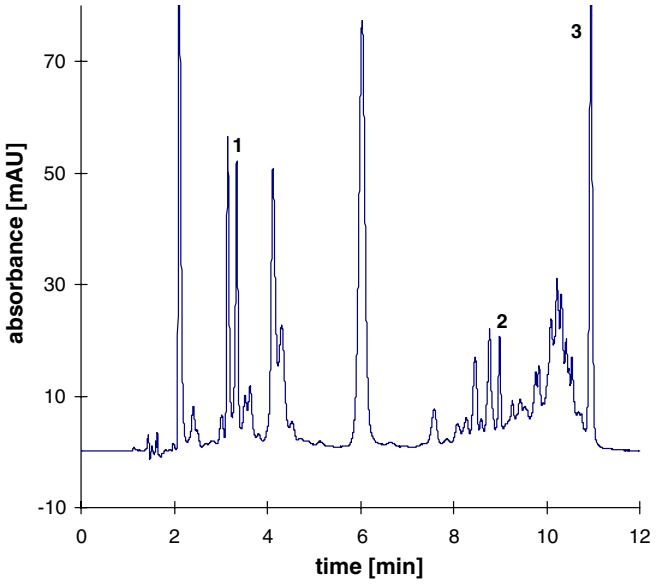

Fig. 3. Typical chromatogram of orange juice after reduction and derivatization with CMQT. Peaks: **1** – glutathione (61.0 μmol L^{-1}); **2** – cysteine (4.1 μmol L^{-1}); **3** – CMQT excess. Chromatographic conditions are described in the text.

Table 1
Imprecision and recovery study for reduced and total glutathione and cysteine in orange juice ($n = 3$)

Thiol	Added (μmol L^{-1})	Measured ± SD (μmol L^{-1})	Imprecision RSD (%)	Recovery (%)
Reduced glutathione	0	21.7 ± 1.3[a]	5.9	–
	20	42.5 ± 0.8	2.0	101.8
	70	92.7 ± 3.5	3.7	101.1
Total glutathione	0	49.1 ± 4.4[a]	9.0	–
	80	136.2 ± 8.4	6.2	100.2
	160	209.6 ± 15.3	7.2	100.2
Reduced cysteine	0	3.0 ± 0.1[a]	4.2	–
	6	8.9 ± 0.2	2.1	99.1
	10	13.2 ± 0.3	2.6	101.3
Total cysteine	0	7.4 ± 0.3[a]	3.8	–
	20	27.4 ± 0.6	3.4	99.9
	40	47.1 ± 1.0	2.2	100.2

[a] Endogenous concentrations.

expressed as relative standard deviation values (RSD) for three concentrations: zero point (endogenous concentration), the center and the upper boundary of the standard curve, were studied and results are inserted in Table 1. With no outliers excluded, the imprecision was for GSH, GSSG, CSH and CSSC within 2.0–5.9%, 6.2–9.0%, 2.1–4.2% and 2.2–3.8%, respectively. Analytical recovery for analytes was from 99.1% to 101.3%.

Table 2
Reduced and total glutathione and cysteine profiles of fruit juices

Juice	Total	Reduced	% of Total
Glutathione (μmol L^{-1})			
Orange juices			
Cappy[a]	49.1 ± 4.4	21.7 ± 1.3	44.2
Clippo[a]	98.4 ± 1.4	64.0 ± 4.0	65.0
Hortex[a]	80.1 ± 4.2	46.6 ± 1.5	58.1
Fortuna[a]	45.1 ± 1.1	28.6 ± 0.1	63.4
Riviera[a]	88.2 ± 2.8	47.3 ± 0.5	53.6
Neat orange juice	247.3 ± 6.7	192.3 ± 1.1	77.8
Grapefruit juices			
Hortex (red)[a]	23.6 ± 0.8	9.4 ± 1.5	40.0
Hortex (yellow)[a]	12.7 ± 0.2	2.2 ± 0.0	17.1
Neat juice (red)	51.2 ± 1.6	41.5 ± 1.4	81.0
Neat juice (yellow)	78.7 ± 6.0	60.8 ± 8.9	77.3
Cysteine (μmol L^{-1})			
Orange juices			
Cappy[a]	7.4 ± 0.3	3.0 ± 0.1	40.5
Clippo[a]	8.7 ± 0.1	3.9 ± 0.0	45.1
Hortex[a]	9.9 ± 1.1	4.0 ± 0.1	41.1
Fortuna[a]	4.5 ± 0.1	1.6 ± 0.0	35.5
Riviera[a]	5.8 ± 0.1	1.4 ± 0.0	24.0
Neat orange juice	19.5 ± 0.9	15.1 ± 0.4	77.9
Grapefruit juices			
Hortex (red)[a]	3.4 ± 0.2	Not detected	–
Hortex (yellow)[a]	2.5 ± 0.0	Not detected	–
Neat juice (red)	15.3 ± 0.1	14.9 ± 1.4	97.1
Neat juice (yellow)	36.7 ± 2.9	22.1 ± 7.0	60.3

[a] Commercially available soft drinks.

3.4. Application to fruit juice samples

The proposed method was applied for the determination of reduced and total glutathione and cysteine in fresh, neat orange and grapefruit juices and in few commercially available soft drinks. Samples were prepared according to the recommended procedure and after centrifugation analyzed chromatographically. Total glutathione was higher in orange than in grapefruit juice, but total cysteine was higher in grapefruit than in orange juice. In general aminothiol concentrations were higher in fresh than in commercially available juices. Yellow grapefruit were much richer in cysteine and glutathione than red ones. Detailed results are shown in Table 2.

4. Conclusion

The analytical procedure described in this paper for the determination of glutathione and cysteine was based on derivatization of the thiol group with 2-chloro-1-methylquinolinium tetrafluoroborate – as a derivatization reagent, previously used for the quantitation of plasma (Bald, Chwatko, Głowacki, & Kuśmierek, 2004), urinary (Kuśmierek, Głowacki, & Bald, 2006) or saliva thiols (Bald & Głowacki, 2005). Because, the bulk of thiols occur in the disulfide forms rendering them inaccessible to derivatization reagent, and in order to determine their total contents, disulfide bonds must be cleaved with suitable reducing reagent to liberate a free thiol. For this purpose sodium borohydride was used. The proposed method was satisfactorily applied to reduced and total glutathione and cysteine measurement in the orange and grapefruit juices. The results showed that the thiol levels were higher in fresh juices than in commercially available soft drinks (Table 2). Valencia, Martin, and Hardy (2001) reported that fresh fruits and vegetables and freshly cooked meats are high in GSH. Frozen foods generally have GSH content comparable to that in fresh foods, but other forms of preservation usually result in substantial or complete loss of GSH (Jones et al., 1992). Moreover, normal cooking results in variable loss, typically with different consequences for plant and animal products (Valencia et al., 2001). Therefore, these differences in thiol levels in fresh and in commercial drinks may be associated with preservation of juices in manufacturing process, or more probably, with dilution process which facilitate the oxidation. The high levels of thiols and the high GSH-to-GSSG ratio confirm that these juices (especially fresh) are desired components of the human diet.

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