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Stability of Phenolic Compounds Isolated from Cocoa, Green Tea and Strawberries in Hank's Balanced Salt Solution under Cell Culture Conditions

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The study analysed the stability of polyphenols present in extracts isolated from cocoa, green tea and strawberries in Hank's Balanced Salt Solution buffers. The extracts were incubated under conditions commonly used for intestinal absorption experiments with human epithelial cells. The polyphenols were analysed with HPLC-DAD-FLD-MS method. The concentrations of most of the polyphenols monitored changed during 2 h of incubation. The flavan-3-ols of cocoa exhibited similar stability and all were more stable at pH 6.5 than 7.4. The concentration of procyanidin B2 at pH 7.4 decreased to the highest extent. Green tea predominant catechins: epigallocatechin gallate and epigallocatechin, were very vulnerable in HBSS both at pH 6.5 and 7.4; their specific molecular structure may be responsible for this phenomenon. The anthocyanins of a strawberry extract were more stable at pH 6.5. The considerable increase in ellagic acid concentration at pH 7.4 might be explained by possible degradation of other compounds of the extract. It is highly recommended to perform stability tests before transport experiments in a cell culture model in order to avoid misinterpretation of results.

INTRODUCTION

Polyphenols exhibit a wide range of biological activities, including antioxidant, anti-cancerogenic, cardioprotective, antimicrobial and neuro-protective properties [Jaganath Crozier, 2010]. Regular consumption of polyphenol-rich foods was associated with the diminished risk of degenerative diseases like cancer, atherosclerosis and diabetes [Andlauer *et al.*, 1998]. In this context the issue of the absorption pathways of phenolic compounds is of vital importance. One of the approaches applied to study this topic is a cell culture model of human intestinal absorption. Initially, human epithelial cell lines as a tissue culture model for permeability measurements were used only in drug discovery process [Artursson *et al.*, 1996; Hubatsch *et al.*, 2007; Tanaka *et al.*, 1995; van Breemen & Li, 2005]. The cell culture models were proved to be a good alternative for animal studies and have emerged as one of the standard *in vitro* tools to predict *in vivo* intestinal absorption of various substances. Subsequently, they were found to be suitable to study intestinal transport of phytochemicals [Barrington *et al.*, 2009; Deprez *et al.*, 2001], and nowadays, cell line models are called an indispensable tool in food science and nutrition [Langerholc *et al.*, 2011]. The studies of epithelial transport/bioavailability are performed in conditions mimicking physiological conditions, involving pH 6.0–7.4. However, phenolic compounds were reported to be labile at neutral and mildly alkaline pH [Zhu *et al.*, 1997]. They can easily oxidise at pH higher than 6.5. Therefore, it is very important to control the effect of assay conditions, mainly buffers and pH, on the stability of phenolic compounds studied. Their impact cannot be neglected in order to avoid artifacts, and misinterpretation of results [Halliwell, 2011].

The effect of pH conditions on individual standard phenolic compounds such as procyanidin dimers B2 and B5, catechin and epicatechin [Zhu *et al.*, 2002], procyanidin A2 [Lu *et al.*, 2011] as well as catechins isolated from green tea [Hong *et al.*, 2002; Sang *et al.*, 2005; Zhu *et al.*, 1997] was investigated. Some of the above mentioned studies reported also the stability of phenolic compounds in most common media used in cell culturing: McCoy, DMEM or MEM. Long *et al.* [2010] observed the stability of hydroxytyrosol, delphinidin chloride, rosmarinic acid, curcumin, apigenin, naringenin, hesperitin and resveratrol.

Recently the need to consider bioavailability of not only pure compounds but also accompanied by the other compounds, as they appear in food matrixes, has been emphasised [Dupas *et al.*, 2006; Keogh *et al.*, 2007; Neilson & Ferruzzi, 2011]. The effect of co-occurring substances might be also crucial for the stability of phenolic compounds present in plant extracts. Therefore, the present study aimed to examine the stability of phenolic compounds of cocoa, green tea and strawberry extracts in Hank's Balanced Salt Solution

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(HBSS) buffers pH 6.5 and 7.4 under conditions specific for a cell model of intestinal absorption. The plant materials investigated in our study represent sources of different types of phenolic compounds: flavan-3-ol monomers and polymers (cocoa), flavan-3-ols (green tea), anthocyanins and hydrolysable tannins (strawberries).

MATERIAL AND METHODS

Materials

All solvents and reagents were of analytical grade or higher. Acetone and ethanol (absolute) were purchased from Cochimy (Martigny, Switzerland). Dimethyl sulfoxide (DMSO), acetic acid, formic acid, Amberlite XAD-16, Sephadex LH-20, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and magnesium sulphate were obtained from Sigma-Aldrich (Buchs, Switzerland). Methanol, hexane, and acetonitrile of HPLC grade were acquired from Lab-Scan (Gliwice, Poland). Monosodium phosphate, disodium phosphate, potassium chloride, sodium chloride and calcium chloride (suitable for cell culture) were purchased from AppliChem (Darmstadt, Germany). Deionised water was obtained using Milli-Q water purification system (Millipore AG, Zug, Switzerland). Epicatechin (EC), catechin (C), epigallocatechin (EGC), epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) standard compounds were obtained from Extrasynthese (Genay, France). HBSS buffers were prepared in our lab: 8.0 g NaCl, 0.4 g KCl, 1.0 g glucose, 0.05 g Na_2HPO_4, 0.054 g KH_2PO_4, 0.19 g $CaCl_2$, 0.1 g $MgSO_4$, 0.35 g $NaHCO_3$, 2.38 g HEPES was dissolved in 1 L of deionised water and pH was adjusted to the value of 6.5 or 7.4. Green tea extract was purchased from Zhejiang Orient Tea Development Co., Ltd (Hangzhou, China).

Extraction of phenolic compounds

Cocoa defatted powder was purchased from a local supermarket (Sion, Switzerland). The extract of cocoa phenolic compounds was obtained as described elsewhere [Kosińska & Andlauer, 2012]. Briefly, cocoa defatted powder was soaked overnight in a mixture of acetone/water/acetic acid (70/29.5/0.5; v/v/v) at room temperature in darkness at solid to solvent ratio of 1:8. Phenolic compounds were then extracted three times using sonication at 50°C for 10 min. The obtained extract was re-extracted with hexane in order to remove residual lipids. Then, the acetone was evaporated at 50°C under reduced pressure using a rotavapor (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) and purified on Sephadex LH-20. The samples were loaded on the column and washed with 30% methanol to remove sugars and methylxanthines. The compounds of interest were eluted from the column with 70% aqueous acetone (v/v) then solvent was evaporated and water residue was freeze-dried resulting in obtaining a cocoa powder purified extract.

Strawberries (variety Charlotte) were obtained from ACW research center (Agroskop Changins-Wädenswil, Switzerland). They were harvested at commercial ripeness and frozen immediately at -20°C. A strawberry puree was prepared with a Büchi Mixer B-400 (Fa. Büchi, Essen, Germany). They were processed with a rotation speed of 9,000 rpm for 3–5 s. The puree was freeze-dried (Lyolab B II, LSL Secfroid, Switzerland). In order to extract the bioactive compounds a solvent mixture (12 mL per g of strawberry powder) consisting of acetone, water and formic acid (70/29.9/0.1, v/v/v, pH=4.02) was used. The homogenisation was performed with an Ultra Turrax T25 (Ika Labortechnik, Staufen, Germany) at 20,500 rpm for 3 min. Then, after centrifugation (10 min, 5,000×*g*, 5°C), the supernatant was collected and the precipitate was homogenised with the solvent again. The procedure was repeated two times. The collected solvent was evaporated immediately (Laborota 40M-digital). Then the hydrophilic compounds such as sugars and organic acids were removed from the aqueous residue by a separation on a column filled with Amberlite XAD-16. The polyphenols were eluted with aqueous ethanol, and solvent was evaporated at 40°C. The aqueous residue was freeze-dried.

Stability analyses

The strawberry and cocoa extracts were pre-solubilised in DMSO and then dissolved in HBSS at two different pH values: 6.5 and 7.4 at a final concentration of 5 mg/mL and 1 mg/mL, respectively. The final concentration of DMSO was 1%. The green tea extract was directly dissolved in an HBSS buffer to provide the concentration of 1 mg/mL. Freshly prepared solutions were incubated at 37°C in a humidified atmosphere of 5% CO_2. The samples were collected at time intervals up to 2 h and immediately stabilised with addition (9:1 ratio) of a mixture of 1% ascorbic acid and 0.28% H_3PO_4 [Zuo *et al.*, 2006]. Aliquots were filtered (0.45 μm) and phenolic compounds were analysed by HPLC. The results presented are the means of three individual incubations ± standard deviation.

Analysis of phenolic compounds by HPLC-DAD-FLD-MS

An Agilent 1200 series liquid chromatograph (Agilent Technologies, CA, USA) comprised of an autoinjector, a quaternary pump, a column counterpart, a UV-DAD and fluorescence detectors (FLD) were employed. A 20-μL portion of the sample was injected onto a C18 column (250 × 4.6 mm i.d., particle size 5 μm, Phenomenex, Torrance, CA, USA) with a security C18 guard column (4×2.0 mm i.d., 5 μm, Phenomenex) at a flow rate of 0.7 mL/min. The mobile phase was composed of three solvents: A (H_2O), B (acetonitrile) and C (1% aqueous formic acid). A linear gradient elution was adjusted individually for separation of phenolic compounds from cocoa, strawberries and green tea, and gradient mobile phase compositions were presented in Table 1. The column temperature was set at 30°C and compounds were monitored at a wavelength of 280, 360 and 500 nm. Fluorescence detection was preformed with excitation at 276 nm and emission at 316 nm. Standard solutions of EGC, C, EC, EGCG, ECG were injected and calibration curves were constructed for quantification. Gallocatechin gallate (GCG) was quantified as EGCG, and procyanidin B2 as catechin equivalents. In the case of strawberry phenolic compounds the change in concentration in the course of time was calculated as the ratio of the peak area to their initial value. For identification of individual compounds mass spectra were acquired using a ThermoQuest-Finningan LC Q DECA with electrospray ionization (ESI) in the negative and positive modes in the range of *m/z* 100–2 500. The capillary tempera-

TABLE 1. Composition of gradient mobile phases for HPLC separations.

	Solvent A (H_2O)	Solvent B (acetonitrile)	Solvent C (1% aqueous formic acid)
Cocoa			
0–14 min	85–75%	5–15%	10%
14–43 min	75–65%	15–25%	10%
43–45 min	65–5%	25–85%	10%
Green tea			
0–14 min	85–75%	5–15%	10%
14–43 min	75–65%	15–25%	10%
43–45 min	65–5%	25–85%	10%
45–50 min	5%	85%	10%
Strawberry			
0–10 min	80–72%	10–18%	10%
10–15 min	72%	18%	10%
15–30 min	72–65%	18–25%	10%
30–31 min	65–5%	25–85%	10%
31–40 min	5%	85%	10%

ture was 275°C, sheath gas flow was 45 units/min. The source voltage was set at 4 kV.

RESULTS AND DISCUSSION

HBSS is the most commonly employed buffer for transport experiments performed in cell culture models [Hubatsch *et al.*, 2007]. The two distinct pH values 6.0–6.5 and 7.4 are often utilised to form pH gradient between apical and basolateral compartments of Transwell system to mimic conditions peculiar for intestine/blood barrier [Tian *et al.*, 2009]. In our study we investigated the stability of phenolic compounds of cocoa, green tea and strawberry extracts in HBSS buffers pH 6.5 and 7.4 under cell culture conditions. The samples were incubated for 2 h as most of transport experiments are conducted for this time.

The predominant compounds of the cocoa extract investigated in our study were (+)-catechin, (-)-epicatechin and procyanidin B2. The changes in the concentration of those compounds during incubation at two different pH values were observed and illustrated in Figure 1. Generally, the compounds were more stable at pH 6.5 than 7.4. The stability of all three flavan-3-ols observed was similar. The highest loss, amounting to 22%, was noted in the case of procyanidin B2 at pH 7.4. In turn, the most stable compound was (+)-catechin at pH 6.5, with the loss of 12%. The studies conducted on pure monomers and dimers isolated from cocoa showed higher stability of monomers than dimers in phosphate buffers both at pH 6.0 and 7.4 [Zhu *et al.*, 2002]. At pH 6.0 monomers remained almost intact for 24 h whereas degradation of dimers up to 20% was observed. At pH 7.4 the authors observed large degradation of monomers, only 20% remained after 24 h. On the other hand, in the case of dimers only 40% of dimer B5 remained after 2 h and slower, but also extensive degradation of dimer B2 occurred. The degradation of procyanidin A2 in DMEM was even more rapid, with half-life of only about 15 min [Lu *et al.*, 2011].

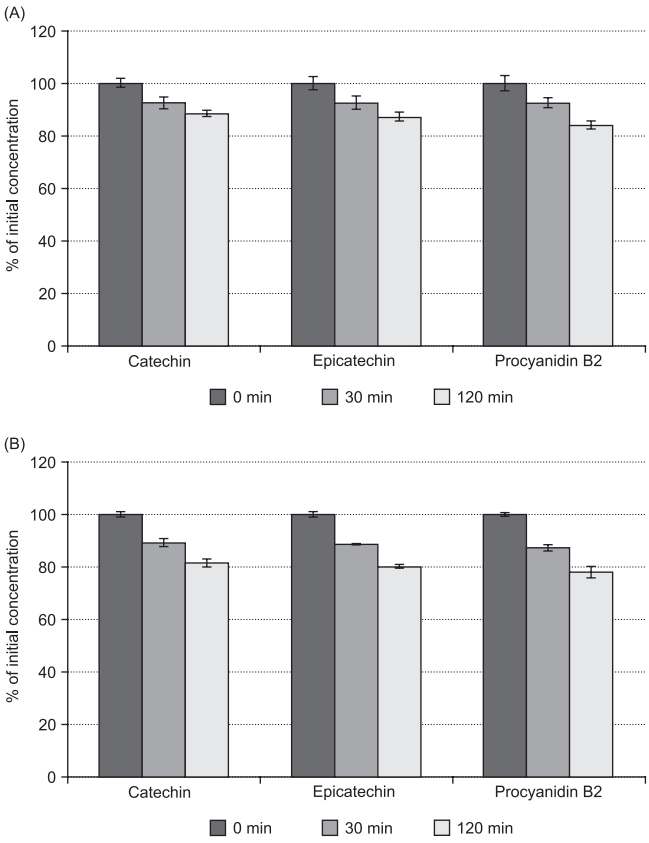

FIGURE 1. Stability of predominant compounds of cocoa extract in HBSS buffer at pH 6.5 (A) and 7.4 (B) during incubation at 37°C.

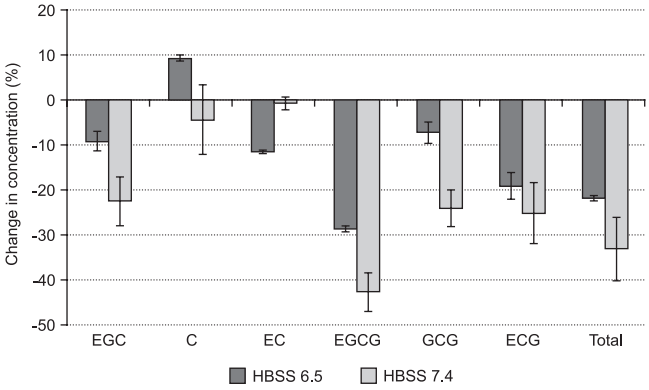

FIGURE 2. Change in the concentration of green tea extract catechins at t=0 min in HBSS buffers pH 6.5 and 7.4 compared to their concentration in distilled water (EGC, epigallocatechin; EC, epicatechin; EGCG, epigallocatechin gallate; GCG gallocatechin gallate; ECG, epicatechin gallate, total is a sum of above listed catechins).

In the green tea extract six catechins: epicatechin (EC), catechin (C) epigallocatechin (EGC), epigallocatechin gallate (EGCG) gallocatechin gallate (GCG) and epicatechin gallate (ECG), were identified. Changes in their concentration in two different HBBS solutions were monitored. Surprisingly, an extensive decrease of green tea catechins concentration in HBSS buffers occurred immediately after dissolving, comparing to the same amount of green tea extract dissolved in distilled water (Figure 2). EGCG was the most susceptible

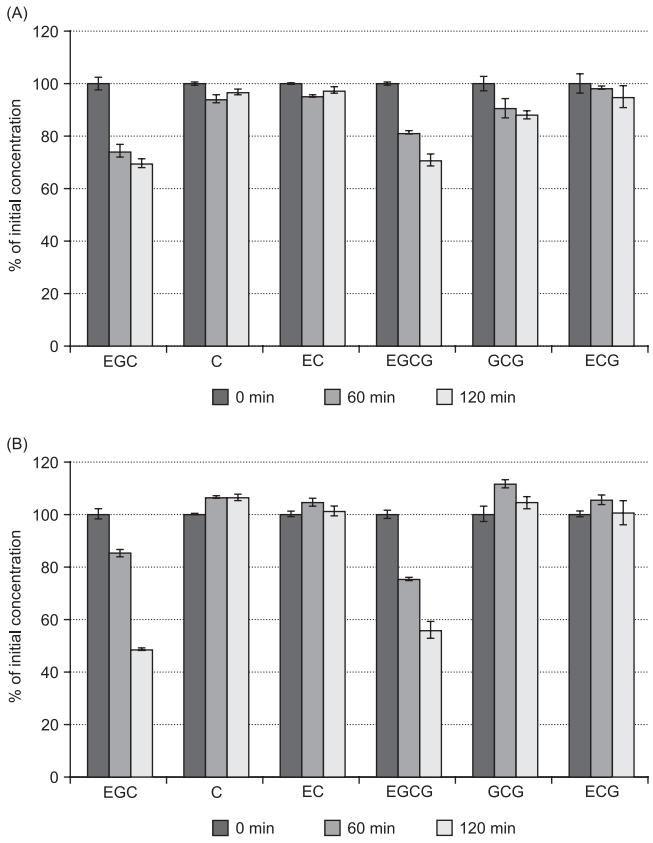

FIGURE 3. Stability of green tea extract catechins in HBSS buffer at pH 6.5 (A) and 7.4 (B) during incubation at 37°C (EGC, epigallocatechin; EC, epicatechin; EGCG, epigallocatechin gallate; GCG gallocatechin gallate; ECG, epicatechin gallate, total is a sum of above listed catechins).

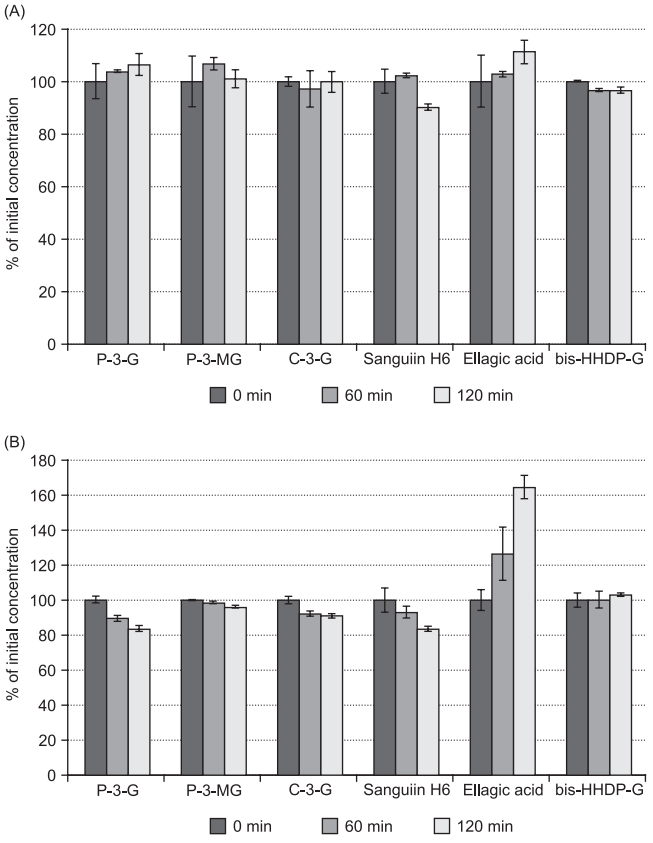

FIGURE 4. Stability of predominant compounds of strawberries extract in HBSS buffer at pH 6.5 (A) and 7.4 (B) during incubation at 37°C (P-3-G, pelargonidin-3-glucoside; P-3-MG, pelargonidin-3-malonylglucoside; C-3-G: cyanidin-3-glucoside; bis-HHDP-G, bis-hexahydroxydiphenoyl-glucose).

to degradation in HBSS at both pH 6.5 and 7.4 with a loss of 29% and 43%, respectively. High degradation in HBSS at pH 6.5 and 7.4 of GCG (16% and 31%), ECG (19% and 25%) and EGC (9% and 22%) were also observed, while C and EC were more stable in HBSS with only slight changes noticed.

The observation of catechins stability in HBSS at pH 6.5 and 7.4 in the course of time showed further degradation of EGC and EGCG (Figure 3). And thus, 30% and 29% loss of EGC and EGCG, respectively was observed during incubation in HBSS at pH 6.5 after 2 h. Both compounds were even more labile in HBSS at pH 7.4, the loss of 52% and 44%, for EGC and EGCG, respectively was observed after 2 h. Moreover, the decomposition of both compounds was time--dependent. In contrast to EGC and EGCG, the other catechins, *i.e.* C, EC, GCG and ECG were quite stable in HBSS buffers with slight changes during 2 h incubation. It is worthy of notice that contrary to EGC and EGCG, the other four catechins were more susceptible at lower pH 6.5 than at higher pH 7.4.

The relative stability of tea catechins was previously reported to be in the following order: EC>ECG>EGCG>EGC [Neilson & Ferruzzi, 2011]. In our study, EGCG was more stable than in previously reported studies mentioning a half time of EGCG at 30 min [Sang *et al.*, 2005]. Our results are in line with the other study, in which the authors described the approximate half-time of EGCG in Tris–HCl buffer at pH 7.2 as 2 h [Sang *et al.*, 2007]. Hong *et al.* [2002] monitored stability of EGCG in HBSS buffer at pH 7.4 and detected oxidative products after 10 min of incubation. Newly formed compounds were larger and more hydrophobic. After MS analysis they were identified as theasinensin and another product with molecular weight of 884.

The rapid degradation of EGC and EGCG should arouse attention. EGCG and EGC were more vulnerable in HBSS both at pH 6.5 and 7.4, their specific molecular structure may be responsible for this phenomenon. Both EGCG and EGC have an additional hydroxyl group at position 5' compared with ECG and EC. The three adjacent hydroxyl groups at position 3', 4', and 5' in EGCG and EGC were more vulnerable to oxidative decomposition than the two adjacent hydroxyl groups at position 3' and 4' in ECG and EC [Zhu *et al.*, 1997]. GCG has a similar structure as EGCG, but the content of GCG did not change considerably, with 12% decrease at pH 6.5 but 4% increase at pH 7.4. At high temperatures (98°C, 120°C), the conversion from EGCG to GCG was reported [Chen *et al.*, 2001]. We can surmise that maybe also in HBSS buffer epimerisation of EGCG occurred, and it resulted in a high content of the remaining GCG. Previous reports showed that EC and C might undergo epimerisation at the position 2 to yield C and EC, respectively in simulated intestinal juice [Zhu *et al.*, 2002].

In the case of strawberries we observed the stability of two classes of phenolic compounds characteristic for strawberry fruits: anthocyanins and ellagic acid derivatives.

The strawberry extract contained mainly pelargonidin-3-glucoside (P-3-G), pelargonidin-3-malonylglucoside (P-3-MG) and cyanidin-3-glucoside (C-3-G), as well as sanguiin H6, ellagic acid (EA) and bis-hexahydroxydiphenoyl-glucose (bis-HHDP-glucose). During 2 h of incubation in HBSS buffer at pH 6.5 the concentration of anthocyanins did not change significantly (Figure 4A). On the other hand, in mild alkaline conditions, at pH 7.4 their concentrations decreased. In the case of P-3-G and C-3-G the changes in the concentration were observed after 1 h already (Figure 4B). In the conditions applied, P-3-MG seemed to be more stable than P-3-G. After 2 h 96% and 84% of the initial concentration of P-3-MG and P-3-G, respectively remained intact. The effect of the different pH conditions (6.5 and 7.4) became even more evident after 24 h of incubation. Only 55% of the anthocyanins were still present in the solution at pH 7.4, whereas between 85% and 90% of the anthocyanins remained stable at pH 6.5 (data not shown). It is well-known that anthocyanins are more stable under acidic conditions. The results presented in this study are consistent with those reported by Dai *et al.* [2009], showing a maximum decrease in the concentration of anthocyanins (of raspberry) in two different media (PBS + 10% FBS and RPMI 1640 medium) at pH 7.4 and a temperature of 37°C.

A decrease in the concentration of sanguiin H6 at pH 7.4 was higher than at pH 6.5 within 2 h, however, at pH 6.5 a decrease in the concentration between 1 and 2 h of incubation could be noticed (Figure 4). At pH 7.4 no sanguiin could be detected after 24 h (data not shown). Figure 4B shows a significant increase in EA concentration. The concentration of bis-HHDP-glucose seems to be more stable at pH 7.4 than at pH 6.5. Sanguiin H6 can be hydrolysed very rapidly in slightly alkaline pH, however, it seems to be more stable under acidic conditions [Larrosa *et al.*, 2010]. The considerable increase in EA concentration at pH 7.4 might be explained by possible degradation of sanguiin H6. Additionally, literature data reports the presence of other EA releasing compounds in strawberries, like galloyl-HHDP-glucose and bis-HHDP-glucose [Aaby *et al.*, 2007; Kajdzanoska *et al.*, 2010]. An increase in the concentration of EA due to hydrolysis of ellagitannins was also reported to occur in the human small intestine [González-Barrio *et al.*, 2010]. The concentration of bis-HHDP-glucose which possess a similar structure as sanguiin H6 (dimer of galloyl-bis-HHDP-glucose), seems to be more stable in the HBSS solution (Figure 4). Analogically, it might be explained by the degradation of sanguiin or galloyl-bis-HHDP-glucose and the following releasing of bis--HHDP-glucose fragments.

CONCLUSIONS

Considering the obtained results we can conclude that majority of phenolic compounds might undergo oxidation in buffers of biological relevance, especially at slightly alkaline conditions. It seems that phenolic compounds present in the extracts might be protected by co-occurring substances, since the degradation noticed was lower comparing to results presented by other authors for pure compounds. However, in order to avoid misinterpretation of the results the stability of compounds of interest in buffers employed should always be monitored before experiments in cell culture are performed. Otherwise, the observation ascribed to cells activity might be in fact evoked by pH conditions.

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