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Biological activity and sensory evaluation of cocoa by-products NADES extracts used in food fortification

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ABSTRACT

Novel approach in food fortification by using natural deep eutectic solvent (NADES) was studied. Cocoa by-products were chosen as sustainable source of polyphenols and extracts were prepared following the green extraction principles by using NADES. Antioxidative and biological activities of prepared extracts rich in polyphenols were determined and they were used for fortification of chocolate milk. Furthermore, electronic tongue analysis combined with SIMCA multivariate data analysis was used for the first time, to evaluate the sensory acceptability of chocolate/cocoa drinks with addition of NADES extracts, which is after safety issues, surely one of the most important features for possible application of those extracts in food industry. Based on presented results it is evident that carefully selected NADES could be used for efficient extraction of polyphenols from cocoa by-products and that obtained NADES extracts could be used for fortification in food industry, without removal of extraction solvent, since they are proven safe and estimated as sensory acceptable.

1. Introduction

The increased awareness of present day consumers about possible health benefits of their diet has led to an increased demand for foods containing biologically active compounds. As a consequence, the new challenge in food industry is to enrich their products for human consumption with compounds having functional properties like antioxidants. Specifically, polyphenols gain much interest due to their potential positive effects on human health, such as anticancer, antioxidant and anti-inflammatory activity (Teplova et al., 2018). Accordingly, research in food science, is focused to find new and sustainable sources of antioxidants, optimizing the extraction and purification methods as well as developing innovative functional foods that promote health (Caleja, Ribeiro, Barreiro, & Ferreira, 2017; Cory, Passarelli, Szeto, Tamez, & Mattei, 2018). However, the first step in development of functional food enriched with biologically active compounds is the extraction of the target compounds. This is often related to shortcomings like high organic solvent consumption, high energy consumption, and large quantities of waste materials. To overcome those shortcomings, the use of new, green and sustainable technologies is recommended (Chemat et al., 2019).

Herein we report an efficient procedure for polyphenols extraction addressing the principles of green processing. In general, green extraction is based on the discovery and design of extraction processes which reduce energy consumption, and use alternative green solvents and renewable natural products, that ensure a safe and high-quality extract/product (Chemat, Vian, & Cravotto, 2012). For example, one of the interesting approaches to obtain polyphenolic rich extracts is the exploitation of food wastes since food processing generates a substantial volume of solid organic by-products, which are still rich in polyphenols (Chemat et al., 2012). Also, the use of innovative extraction methods such as microwave and ultrasound technology is recommended in order to reduce energy consumption and improve extraction efficiency (Chemat et al., 2012). Consequently, there is a growing area of research for the development of green extraction techniques. This research aims at the design of new, environmentally friendly and tuneable solvents that can meet both the technological and economic demands (Cvjetko Bubalo, Vidović, Radojčić Redovniković, & Jokić, 2015; Cvjetko Bubalo, Vidović, Radojčić Redovniković, & Jokić, 2018).

One of the promising types of solvents for efficient extraction of natural compounds from plants are natural deep eutectic solvents

Abbreviations: NADES, natural deep eutectic solvents; HBD, hydrogen bond donor; HBA, hydrogen bond acceptor

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(NADES). NADES are formed from simple, cheap, safe and naturally occurring compounds with a high safety profile. Most NADES building components have GRAS status (Radošević et al., 2018). Furthermore, it has been shown that the solubility of a number of naturally occurring compounds increased several orders of magnitude in NADES, when compared with the commonly used organic solvents (Cvjetko Bubalo et al., 2018; Zainal-Abidin, Hayyan, Hayyan, & Jayakumar, 2017). Higher solubility in these solvents is explained by the formation of hydrogen bonds between compounds of interest and solvent components (Dai, van Spronsen, Witkamp, Verpoorte, & Choi, 2013). There are two main benefits for application of NADES as a solvent for extraction of natural compounds. Firstly, NADES are highly selective and could extract both polar and nonpolar compounds and secondly and maybe even more importantly, stability of target compounds in NADES is better than in conventional solvents (Dai et al., 2013). Based on all mentioned aspects, it seems that extracts prepared by NADES could be applied in food industry. The use of NADES extracts, as such, without the need of downstream purification of the target compounds, would avoid the need of further extensive downstream operation as in conventional extraction processes, and consequently reduce the use of environmentally unfriendly organic solvents (Dai et al., 2013; Radošević et al., 2016). This approach would be interesting for preparation of ready to use extracts which could be used for food fortification. Also, this approach would satisfy several commercial demands such as prolonged stability of polyphenols, as well as no need for downstream purification which is one of the highest costs in industrial extraction process.

To the best of our knowledge, there are no examples in literature of such innovative approach in food fortification which studies safety issues, as well as sensory analysis. Both are important features which support the thesis of applying NADES extracts in final food products. Currently, only Naturex produce commercially available NADES-based extracts which are used in cosmetics and present on the market (Naturex, Avignone, France).

To test the possibility of food fortification with cocoa by-products different NADES were selected as possible sustainable solvents for polyphenols extraction from cocoa waste products, following green extraction principles. Biological activity of the NADES extracts rich in polyphenols was evaluated and subsequently used for fortification of chocolate milk with polyphenols. Furthermore, electronic tongue analysis was performed and SIMCA multivariate data analysis was used to evaluate the sensory acceptability of chocolate/cocoa drinks fortified with a NADES extracts. It is expected that fortification of food with NADES based extracts would be sensory acceptable for human consumption.

2. Methods

2.1. General

Choline chloride, betaine, citric acid, glycerol, glucose epicatechin, catechin, procyanidin B1, procyanidin B2 and protocatechuic acid were purchased from Sigma (St. Louis, MO, USA). Cocoa by-products were obtained from the University of Turin.

Software Statistica V.12 (Statsoft Inc., Tulsa, OK, USA) was used for the statistical analysis of all experimental results.

2.2. Preparation and characterization of the natural deep eutectic solvents

Choline chloride (Ch) was dried in a vacuum concentrator (Savant SPD131DDA SpeedVac Concentrator, Thermo scientific, USA) at 60 °C for 24 h before use. The NADES were synthesised at certain molar ratios of Ch to hydrogen bond donor (HBD) with addition of 30% of water as described in Panić, Gunjević, Cravotto, and Radojčić Redovniković (2019a). NADES forming components were placed in a round-bottomed glass flask and were stirred at 50 °C for 2 h. Prepared NADES were choline chloride:citric acid (2:1, ChCit), choline chloride:glycerol (1:2, ChGly), Choline chloride:glucose (1:1, ChGlc), betaine:citric acid (1:1, BCit), betaine: glycerol (1:2, BGly) and betaine:glucose (1:1, BGlc). NADES abbreviations and corresponding mole ratios are given in Table S1.

The pH values of NADES were determined using a 405-DPAS pH-electrode (Mettler Toledo, Zagreb). The polarity of NADES was determined using Nile red as a solvatochromic probe, as described in Jeong et al. (2017). Viscosity was measured according to Mitar et al., (2019).

2.3. Preparation of NADES-based extracts rich in polyphenols

Extraction was performed in a US bath XUB5 (XUB Series Digital Ultrasonic Baths, BioSan, Latvia), equipped with Digital LCD controls, a timer and a heater (Heater power 150 W) (Cvjetko Bubalo et al., 2018). Solid-liquid ratios of 0.05 g of whole cocoa beans per mL of prepared NADES (Table S1) or aqueous ethanol (70% of ethanol), were used for extraction. Extractions were carried out under US (power 150 W) at constant temperature (60 °C) for 50 min. Extracts were filtered and supernatant was adjusted to a final volume of 10 mL (0.05 mg mL-1) and stored at +4 °C until further analyses (total phenolic and procyanidins, HPLC analyses and ORAC) were performed. All analyses were conducted in three different samples.

2.4. Total phenolics and total procyanidins analyses

Chocolate milk, before polyphenol analyses were prepared according to Chávez-servín et al. (2015).

Total phenolic content (TP) of cocoa by-products extracts and chocolate milk was determined by Folin-Ciocalteu method as briefly described in Mazor Jolić, Radojčić Redovniković, Marković, Ivanec Šipušić, and Delonga (2011). The absorbance was measured at 760 nm and results were expressed as mg of gallic acid equivalent per g of dw (mg GAE g dw^{-1}).

Total procyanidins were determined by the procedure described by Mazor Jolić et al. (2011). The absorbance was measured on the spectrophotometer GENESYS 10S, (ThermoFisher Scientific, Madison, SAD) at 540 nm. Results were expressed in mg of catechin equivalents per g of dw using the appropriate standard curve (five points from 0.0125 to 1 mg L^{-1} catechin).

2.5. Oxygen radical absorbance capacity assay (ORAC)

ORAC was determined according to the Ninfali, Mea, Giorgini, Rocchi, and Bacchiocca (2005) and results were expressed as relative ORAC values (μmol TE g dw $^{-1}$). The assay was conducted in 3 mL of reaction mixture with 2.25 mL of fluorescein sodium salt (0.04 μmol L^{-1}) in sodium phosphate buffer (0.075 M, pH 7.0), and 0.375 mL diluted extract, Trolox (25 μmol L^{-1}) as standard or 0.075 M sodium phosphate buffer (pH 7) as blank control. The reaction mixtures were incubated for 30 min at 37 °C, and after incubation for 30 min at 37 °C, 0.375 mL of 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) was added. Fluorescence was recorded per min up to value zero by a Varian Cary Eclipse Spectrofluorimeter (Palo Alto, CA, USA) with 485 nm excitation and 520 nm emission.

2.6. HPLC analyses

HPLC analyses were performed on the Agilent 1200 Series HPLC system (Agilent, San Jose, CA, USA) equipped with a diode array detector (DAD) and Phenomenex C18 column (Kinetex 150 mm × 4.6 mm, 2.6 μm, 100 Å), according to Mazor Jolić et al. (2011).

Briefly, the mobile phase consisted of 2.5% acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL min-1. Gradient

elution was as follows: 0–13 min 3% B,13–18 min 9% B, 18–25 min 11% B, 25–45 min 18% B, 45–50 min 30% B and in 50 min 3% B. Chromatograms were recorded at 280 nm. Detection was performed with a photodiode array detector by scanning between 200 and 400 nm. The identification of the compounds was achieved by comparing their UV spectra and retention times of the separated peaks with the retention times of authentic standards. The identified phenolic compounds (−)-epicatechin, (+)-catechin, protocatechuic acid, procyanidin B1 and procyanidin B2, were quantified with an external standard calibration curve. The calibration curves of the standards were made by diluting the stock standards with methanol (five points from 0.5 to 5 mg L^{-1}).

2.7. Determination of in vitro biological activity by cytotoxicity assay

Antiproliferative activity of the extracts from cocoa by-products prepared in NADES and ethanol were evaluated in vitro against two adherent human tumour cell lines by the CellTiter 96® AQueous One Solution Cell Proliferation (MTS) assay according to the manufacturer's instructions with minor modification as described in Panić et al. (2019b). Briefly, HeLa and HaCat cells were cultured in DMEM supplemented with 5% FBS in the incubator with humidified atmosphere and 5% CO2 at 37 °C. Individual experiments to test impact of the prepared extracts on cell proliferation were performed in BioLite 96-wells plates seeded with exponentially growing cells at the concentration (~3 × 10^4 cells per well in 100 μL of media) and incubated for 24 h, after which the treatment was done. Whole cocoa beans extracts were diluted in the culture medium when applied to the cells so the final volume ratio was 1%, 2%, 2.5%, 4% and 5% (*v*/v), while control cells were non-treated cells. Upon 72 h of treatment, MTS reagent was added to each well, and cells were incubated for further 3 h, after which absorbance at 490 nm was measured on the microplate reader (Tecan, Switzerland). Cell viability was expressed as percentage of treated versus control cells. The experiments were performed three times with five parallels for each volume ratio and data were expressed as the means ± S.D.

2.8. Estimation of the sensory acceptability of milk-based chocolate drinks with addition of NADES-based extracts rich in polyphenols

Electronic tongue analysis was used for the estimation of the sensory acceptability of milk-based chocolate drinks with addition of NADES-based extracts rich in polyphenols. Electronic tongue system (Astree 2, Alpha M.O.S.) consisted of seven potentiometric sensors for the application in food analysis, reference Ag/AgCl electrode, autosampler, and electronic unit connected to the computer.

Six different brand samples of milk-based chocolate drinks (marked as CD1–6) and six different brand samples of milk-based cocoa drinks (marked as KD1–6) available on the Croatian market were purchased. All chocolate drink samples and four out of six cocoa drink samples were purchased as ready for consumption. Two cocoa drinks were instant cocoa drinks, which were prepared with milk according to the manufacturer's instructions. Prepared drinks were used to train the electronic tongue to distinguish sensory acceptable and not acceptable drinks, since the used commercial drinks for the training are acceptable for the consumers. To assess influence of the NADES-based extracts on the sensory acceptability, BGlc and ChGlc were added in chocolate drink CD1 at different concentration levels (1%, 5% or 10%, *v*/v).

Prior to the analysis, potentiometric sensors were conditioned in chocolate drink sample until stable signal was obtained. For the analysis, 80 mL of sample was placed in 120 mL beaker which was placed on the autosampler and the analysis was performed during 300 s, with stirring speed 1 and rinsing the sensors with water after each analysis. Four replicate measurements were performed for each sample, while two measurements (RSD up to 10%) were included in the further statistical analysis.

2.9. Data analysis

All experimental results were statistically analysed using Statistica 8 software. Data in the text and tables are expressed as the mean ± standard deviation (± SD), and error bars in the figures indicate the SD. The differences between the means were analysed by the ANOVA test, followed by post-hoc Tukey's test. A significant difference was considered at a p value < 0.05.

Electronic tongue system analysis of the results was performed by Soft Independent Modelling by Class Analogy (SIMCA) method using Astree 2 software version 3.0.1. Obtained model was validated with "leave one out" method (Alpha, 2003).

3. Results and discussion

3.1. Preparation and characterization of NADES-based extracts

Aiming to develop an environment-friendly extraction method for cocoa polyphenols, green extraction principles were followed (Chemat et al., 2012). According to 1st principle, agro-waste from processing of cocoa by-products was used as a source of cocoa polyphenols. Following the 2nd principle of green extraction, natural deep eutectic solvents were used as extraction solvents. Moreover, to improve the extraction efficiency and reduce time of extraction, extraction was performed under the ultrasound irradiation according to 3rd principle of green extraction (Chemat et al., 2019; Cvjetko Bubalo et al., 2018).

In the search of a suitable extraction solvent, different types of NADES containing acid, polyalcohol or sugar as HBD, which considerably differ in physicochemical properties, were tested. Three of the tested NADES were choline chloride based as they are commonly used for extraction due to their excellent extraction efficiency (Cvjetko Bubalo et al., 2018; Panić, Gunjević, et al., 2019a; Panić, Radić Stojković, et al., 2019b). Besides having good extraction efficiency, choline-based NADES are also appreciated for their biological activity. Choline is essential component of the human diet that is necessary for synthesis of acetylcholine, membrane and signalling phospholipid, and function as important methyl donor. A few studies observed positive association between the higher dietary choline intake and reduced risk for some types of cancer (Panić, Radić Stojković, et al., 2019b). On the other side when choline was used as a component of the ready-to-use extract for food fortification, its odour and taste could be unpleasant for consumers (EFSA FEEDAP Panel, 2011; Panić, Radić Stojković, et al., 2019b). Therefore, besides choline-based NADES, we also tested NADES containing betaine as HBA, which have similar characteristics as choline (Mitar, Panić, Kardum, Halambek, & Sander, 2019). For example, comparing the ChCit and BCit, pH value for both of them is < 3, polarity ~50, density ~ 1.2 g cm^{-3}, EC50 < 2000 mg L^{-1} and antioxidant capacity ~1.5 μmol Trolox equivalent $g_{NADES}{}^{-1}$ (Mitar et al., 2019; Panić, Radić Stojković, et al., 2019b). Similar to choline chloride, betaine is also an essential component of the human diet thought to have a protecting effect on various internal organs, decreasing vascular risk factors and preventing chronic disease (Craig, 2004). Furthermore, sweet taste and slight, characteristic odour of betaine presumably would not have negative influence on sensor analysis of food fortified with extracts obtained with betaine-based NADES (Preedy, and Victor R (School of Medicine, King's College LOndon, U, 2015).

In the case of total polyphenols, the highest extraction efficiency was obtained with BGlc ≈ EtOH ≈ ChGlc followed by ChCit ≈ ChGly > BCit > BGly. Highest efficiency of total procyanidins extraction was obtained also with BGlc, followed by ChGlc ≈ ChCit ≈ EtOH > ChGly ≈ BCit > BGly (Table 1). The amount of extracted polyphenols (15.33–22.82 mg $g_{dw}{}^{-1}$) and procyanidins (0.3 to 1.41 mg $g_{dw}{}^{-1}$) differ considerably indicating that this step, selection of suitable NADES, is crucial for getting improved results in comparison to conventional solvents. In our case, results were compared with aqueous ethanol (70%, *v*/v of EtOH in water) which is a classic

extraction solvent for polyphenols (Baharum, Akim, Hin, Hamid, & Kasran, 2016) and it could be concluded that at least a similar polyphenol content can be obtained with NADES. In literature, the similar polyphenol content were reported in various cocoa by-products (from 13 to 69 mg gallic acid equivalent g_{dw}^{-1}depending of by-products type of and location of cocoa growth and solvent system used in extraction) indicating that this is good approach in recovering cocoa by-products polyphenols (Vásquez et al., 2019). Commonly, extraction efficiency correlates with the physicochemical characteristics of NADES (Radošević et al., 2016). In our case, no correlation with pH and viscosity with extraction efficiency was found (Table S1). However, it is notable that NADES containing glucose as HBD showed the best performance. This indicates that inter-molecular interactions between NADES constituents and phenolic compounds play an important role for the solubility (Dai et al., 2013). The phenolic profiles of the NADES extracts were quantitatively analysed by HPLC to determine possible differences in selectivity of the tested solvents. In all extracts procyanidin B1, procyanidin B2, epicatechin and catechin were identified, but as expected, the content of identified polyphenols differed among solvents. The polyphenols profiles in the extracts were in good agreement with the literature (Mazor Jolić et al., 2011). Protocatechuic acid was detected in NADES extracts, especially with BGlc while it was not detected in ethanolic extracts. Worth mentioning is the high selectivity of BGlc toward protocatechuic acid, the main polyphenolic compound in the extract.

Polyphenols are well known as antioxidants and their health-related activities are related to this property (Teplova et al., 2018). Therefore, the antioxidant activity of the prepared extracts was determined by the oxygen radical absorbance capacity (ORAC) method. The ORAC values of the prepared extracts were between 932.20 ± 20.81 and 1191.88 ± 27.03 μmol TE g_{dw}^{-1}, with the best antioxidant activity obtained for the BGlc, followed by ChCit ≈ ChGlc ≈ EtOH > ChGly > BCit ≈ BGly (Table 1). ORAC values are correlated with total phenolic content, as expected, and in agreement with results on phenolic grape skin extracts obtained by NADES (Radošević et al., 2016). Correlation was also found between ORAC and total procyanidins, while there was no significant correlation with catechin, protocatehuic acid and epicatechin indicating that antioxidant properties are related to synergistic effects of different types of polyphenols rather than specific polyphenolic compounds. Because of their extraction efficiency and their antioxidant activity, the extracts obtained by BGlc and ChGlc, were subject for further evaluation in fortification studies of cholate milk with polyphenols.

3.2. Evaluation of biological activity of NADES extracts

Prior to the application of the extracts in food industry it is necessary to test their potential biological activity and also exclude their possible cytotoxicity. In our research, influence of chosen extracts (ChGlc and BGlc) were tested in vitro on HeLa and HaCat cell lines.

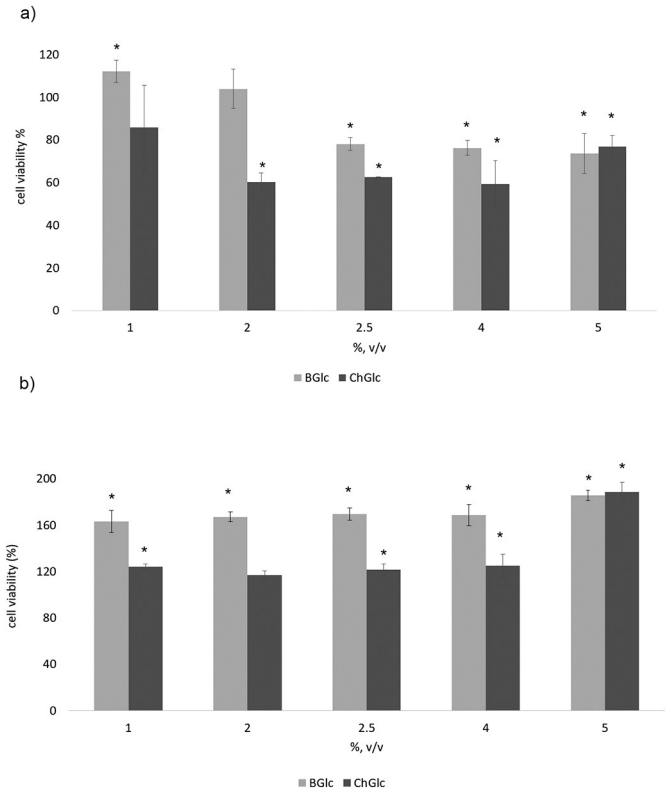

Fig. 1. Effect of prepared extracts on HeLa (a) and HaCat (b) cell viability determined by the MTS assay was assessed in volume ratio 1% - 5% (*v*/v). Cell viability (%) was expressed as percentage of treated cells versus control cells and the data from 3 individual experiments were expressed as the means (n = 5) ± S.D. * statistically different from control.

Considering that NADES remains in the extract, synergistic effect between the solvent and extracted compounds could occur and enhance the antioxidant activity or other biological effects of the final extract (Radošević et al., 2016).

Effect of extracts prepared with ChGlc and BGlc on cell proliferation was evaluated by CellTiter 96® AQueous One Solution Cell Proliferation assay. All samples were applied to cells in different volume ratio 1%-5% (*v*/v) during 72 h (Fig. 1). Both NADES extracts had impact on cell viability of HeLa cells, with a clear effect of the choline-based NADES extract already showed a decrease in viability at 1% (v/v) and at 2–5% cell growth went down with about 40%. The betaine-based NADES extract showed a decrease in viability with about 30% at concentrations from 2.5–5%. Both tested extracts do not show a classical dose–response relationship on HeLa cells, that is, the increase of the concentration does not proportionally decrease the cell viability,

Table 1
Polyphenol content (mg g_{dw}^{-1} of whole cocoa beans) and ORAC values (μmol TE g_{dw}^{-1} of whole cocoa beans) of prepared extract with different NADES. Content of whole cocoa beans were expressed as the means (n = 3) ± S.D. Presented value followed by different lower-case letters (a-f) are significantly different from each other in each group (p < 0.05) as measured by Tukey's HSD test.

Compound	Extracts						
	ChCit	ChGly	ChGlc	BCit	BGly	BGlc	EtOH
Catechin	0.91^{d} ± 0.03	1.11^{c} ± 0.05	0.96^{d} ± 0.04	1.13^{c} ± 0.06	1.46^{b} ± 0.06	2.26^{a} ± 0.06	0.90^{d} ± 0.08
Protocatehuic acid	0.58^{de} ± 0.13	0.70^{d} ± 0.03	0.59^{e} ± 0.06	0.96^{c} ± 0.02	1.45^{b} ± 0.04	2.54^{a} ± 0.04	0.00^{f} ± 0.00
Procyanidins B1	0.52^{b} ± 0.03	0.32^{d} ± 0.01	0.46^{b} ± 0.02	0.17^{e} ± 0.01	0.13^{e} ± 0.04	0.67^{a} ± 0.05	0.39^{c} ± 0.01
Epicatechin	0.33^{e} ± 0.02	0.46^{d} ± 0.02	0.78^{b} ± 0.01	0.18^{f} ± 0.07	0.45^{d} ± 0.07	0.67^{c} ± 0.02	1.02^{a} ± 0.04
Procyanidins B2	0.16^{b} ± 0.01	0.15^{b} ± 0.02	0.14^{b} ± 0.04	0.13^{b} ± 0.06	0.12^{b} ± 0.02	0.86^{a} ± 0.01	0.22^{b} ± 0.03
Total polyphenols	20.34^{b} ± 0.35	19.21^{bc} ± 0.27	21.62^{ab} ± 0.86	18.19^{c} ± 0.52	15.33^{d} ± 0.82	22.82^{a} ± 0.40	22.23^{a} ± 0.20
Total procyanidins	0.74^{b} ± 0.08	0.49^{c} ± 0.04	0.69^{b} ± 0.12	0.3^{c} ± 0.07	0.25^{c} ± 0.45	1.41^{a} ± 0.03	0.63^{b} ± 0.04
ORAC	1180.95^{a} ± 1.82	1024.05^{b} ± 7.29	1168.08^{a} ± 38.33	989.92^{bc} ± 15.92	932.20^{c} ± 20.81	1191.88^{a} ± 27.03	1102.79^{a} ± 9.94

suggesting that probably different compounds present in the obtained extracts contributes to the overall effect on the HeLa cells.

Similar, Radošević et al. (2016) reported a substantial inhibiting effect on cell viability of HeLa cells of grape skin extracts prepared with NADES based on choline chloride and sugars as HBD. Furthermore, polyphenolic extracts from grape and olive pomace, also showed growth inhibition of HeLa cells. Grape pomace extract in ChCit was shown to be the best of all extracts tested, with regard to extraction of total polyphenolic compounds and related biological activities, such as antioxidant and antiproliferative activity (Panić, Radić Stojković, et al., 2019b). Although, results in in-vitro and in-vivo toxicity generally have good correlations, further in-vitro studies would be strongly encouraged. Currently, there is only one in-vivo study on acute toxicity of phenolic NADES extract conducted by Benlebna et al. (2018). These authors concluded that further work is needed to select an efficient and safe dose of a NADES, and a NADES extract, for use in animals or humans. Furthermore, although application of NADES for extraction of polyphenolics is pretty much explored nowadays (Skarpalezos & Detsi, 2019), there is a serious lack of information regarding safety of NADES extracts in-vitro and in-vivo, though toxicity of some pure NADES has been studied. Toxicity of NADES depends on their nature and concentration of the forming components, as well as the model organism on which they are tested. Generally, they are non-toxic, or of low toxicity for those which have organic acids as HDBs (Mitar et al., 2019; Radošević et al., 2016). When it comes to the NADES which were used in this work, they are considered to be non-toxic (Radošević et al., 2015; Radošević et al., 2018). In-vivo acute toxicity of one NADES was assessed by Chen, Wang, Liu, and Zhang (2017) who determined the LD_{50} value of the ChCl-glycerine DES to be was 7733 mg kg^{-1} and concluded that it can be safely administered orally, since it did not promote acute toxicity in rats.

Compared to the influence on the tumour HeLa cell line, the extracts showed a clear growth improving effect of HaCat cells. These immortal keratinocytes, isolated from human skin, when treated with the NADES extracts exhibit an increased proliferation rate, with an increase of the concentration of the NADES extracts. Extract prepared with BGlc showed the highest effect (+60 to +85%) on the proliferation in the range of tested concentrations compared to untreated control cells. The stimulating effect on growth of keratinocytes treated with polyphenols was also reported by Hsu (2003) and Lagha and Grenier (2019). Such an effect of extracts on growth of keratinocytes is interesting in terms of possible applications of those extracts in cosmetic industry, for example in creams for regeneration of damaged skin. That conclusion is supported by research of Macário et al. (2019) who studied cytotoxicity of several NADES against two human skin cell lines, HaCaT32–35 and MNT-136–38, and showed that the ChCl- and tetramethylammonium chloride-containing eutectic solvents were not cytotoxic, and some of them even increased cell viability. That study also indicated that NADES can be safely characterized as “benign”, at least for these cell lines, and could be used for skin-related applications.

At the end, looking at the combined results on tumour HeLa cells and normal HaCat cells, from Fig. 1, it can be concluded that NADES based extracts might have some potential for antitumor activity, since they had negative influence on growth of tumour-, and no or positive effect on normal-cells. Bauer et al. (2016) also reported antitumor activity of extracts prepared from by-products of cocoa production linking that influence with activity of biologically active compounds, i.e. extracted polyphenols. To conclude, the use of NADES in the extractions of polyphenols yields promising results. Nevertheless, the use of these extracts as food supplements, functional food or even nutraceuticals, have to be further investigated by in vivo studies. That is how NADES would be generally accepted as safe solvents for the extraction of e.g. polyphenolics.

3.3. Sensory analysis of chocolate milk fortified with polyphenolic cocoa by-products NADES extracts

Herein, for the first time NADES extracts were used for food fortification, namely polyphenols enriched chocolate milk. From the literature, it is evident that advantages of using NADES-based extract in food industry compared with environmentally unfriendly organic solvents-based extracts will be: (*i*) no downstream purification of polyphenols, (*ii*) higher stability and (*iii*) consequently lower costs of final products (Radošević et al., 2016). However, the focus of developing novel functional foods is not only health benefits, but also sensory properties of the final product must be evaluated. This is the key for further market products acceptability and consequently economic benefit.

For testing, sensory properties of commercial chocolate milk fortified with 1, 5 or 10% NADES extract of cocoa by-products made by either BGlc or ChGlc. The gold standard in the food industry is the sensory analysis of food by human panels (Ross, 2009). However, NADES extract are still not available on the market, and despite that according to the scientific articles NADES extracts are safe for consumption, no legislation is available that claim that NADES are safe for human applications. Therefore, electronic tongue is a good alternative for preliminary sensory analyses and to estimate sensory acceptability of fortified products. The electronic tongue system consists of a set of partially specific, non-selective sensors crudely mimicing human taste receptors and their communication with the human brain (Tudor Kalit, 2014). Results obtained by the electronic tongue have shown good correlation with sensory scores given by human panellists (Escuder-Gilabert & Peris, 2010; Hruškar, Major, & Krpan, 2010). Besides the applicability in the development of new products with yet undefined safety for the consumers, the main advantages of the electronic tongue are sensitivity, speed of analysis, objectiveness, and no sensory fatigue. In the food industry, the electronic tongue combined with various multivariate statistical methods has been applied in process monitoring, shelf-life evaluation, authenticity assessment, food characterization, quality control studies, etc. (Escuder-Gilabert & Peris, 2010; Hruškar et al., 2009; Hruškar et al., 2010; Jiang, Zhang, Bhandari, & Adhikari, 2018). SIMCA, the most used method of the class-modelling techniques, enables building a model with only one group considered as “gold reference” and identifying if unknown samples do or do not belong to the defined group (Berrueta, Alonso-Salces, & Héberger, 2007).

Before extract addition, chocolate milk contained 30 mg of polyphenols per 100 mL (data not shown). By adding only 1% of extract content of polyphenols were at least doubled indicating that this could be a good method for fortification of milk products.

Our study was based on the assumption that consumers consider chocolate/cacao drinks already available at the market sensory acceptable. The SIMCA multivariate analysis method was used to recognize if unknown drinks with addition of the polyphenolic NADES extracts still belong to the well-defined group of commercially available chocolate drinks or not. Samples located within the grey area belong to the previously defined group, while the ones located outside of grey area do not (Fig. 2). Based on the obtained results, most of the samples with the addition of different content of NADES extracts were within the acceptable area. Only exception is a sample with the addition of 10% ChGlc, which is considered unacceptable. It is not surprising that the addition of larger amount of choline chloride based-extracts negatively influences sensory properties of fortified food since it is known that choline chloride possesses specific odour and taste (EFSA FEEDAP Panel, 2011). Furthermore, in case of food fortification with NADES extracts, the amount added of each NADES component should be considered. Though NADES components are abundant in the nature and are part of our daily diet, the recommended daily intake (RDI) has to be taken in account for each component. For example, the recommended daily intake for choline has been set to 550 mg day^{-1} and 425 mg day^{-1} for non-pregnant women. The daily upper limit for

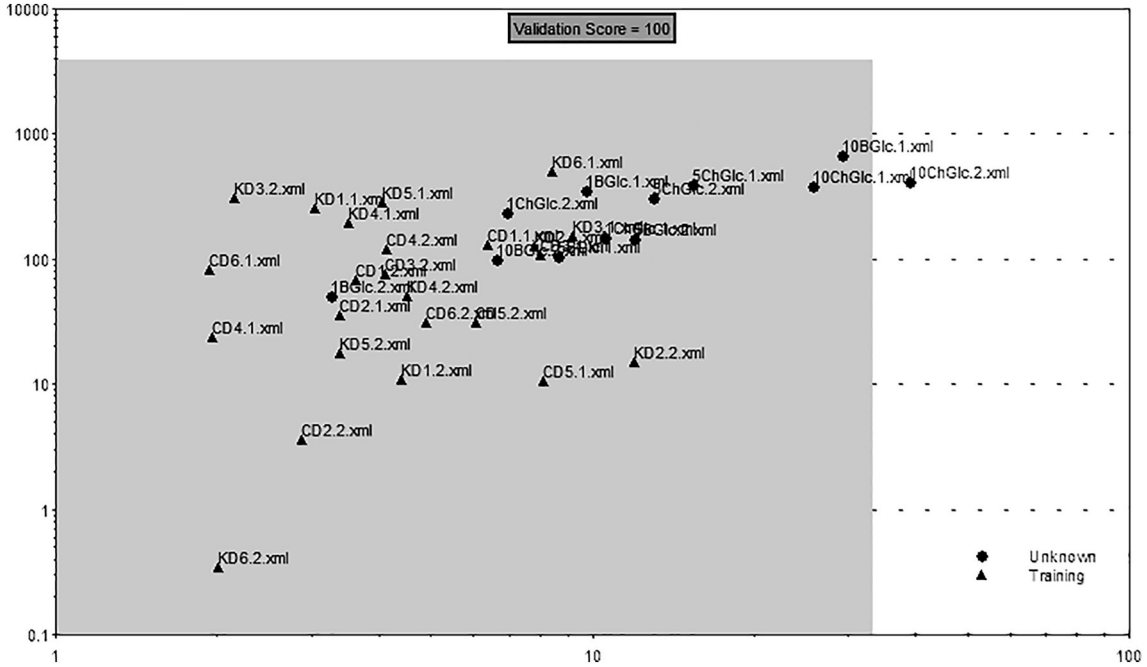

Fig. 2. SIMCA analysis of different brand chocolate/cacao drinks (CD1–6 and KD1–6, respectively) used as training samples and chocolate drink with addition of 1%, 5% and 10% extract in BGlc or ChGlc (1BGlc, 5BGlc, 10BGlc and 1ChGlc, 5ChGlc, 10ChGlc, respectively) defined as unknown samples.

adults is 3500 mg, which is the highest level of intake that is unlikely to cause harm. Similar recommended daily intake is also given for betaine and it is estimated that 400 mg day^{-1} (i.e. 6 mg kg^{-1} body weight (bw) per day for adults) is considered as safe (EFSA FEEDAP Panel, 2019). In our case, with the lowest level of extract (1%) added in 100 mL of chocolate milk, we also added 330 mg of choline chloride and 290 mg of betaine what is lower than RDI. With the highest volume ratio (10%) used for fortification of chocolate milk, 3300 mg of choline chloride was added, what is still lower than upper daily limit for adults. Furthermore, plant matrix used in this study has low polyphenols content and by using another matrix richer in polyphenols, lower volume of NADES could be added. Therefore, matrix with higher polyphenols content, within this approach of fortification, would be recommended. However, in our case, we can conclude that addition of 1%–10% of NADES extracts is sensory acceptable, except from 10% of ChGlc extract, indicating that this approach could be further explored for food fortification in order to ensure consumers acceptability of such new product.

4. Conclusion

Taken all together, this research contributes to additional valorisation and utilization of cocoa by-products in an environmentally friendly way for human consumption. Though, still a lot of work is needed to open the way for NADES, as an extraction solvent in any food, cosmetics and pharmaceuticals related industry. One of the major issues is the establishment of RDI and daily upper limits for NADES itself and NADES forming compounds. Recommendations on those issues as well as proper legislation would surely significantly foster applications of NADES in the mentioned industrial fields. It would be particularly welcomed to establish a database of NADES with corresponding quantities which could be safely used in food industry considering its cytotoxicity, recommendation of daily intake and sensory properties. Herein, the sensory analysis of chocolate milk fortified with polyphenolic cocoa by-products NADES extracts was done for the first time. Based on that, it would be further interesting and useful to evaluate contribution of different NADES to intensify particular tastes, such as sweet, bitter, and sour, and correlate it with conventional sensory analysis by human panels in order to obtain clear evidence which NADES would be suitable for specific food fortification. Therefore, this work presents a valuable step forward to obtain guidance for the legislation and application of NADES plant extracts, as a natural and safe formulation for diverse applications in food industry as well as in cosmetics and pharmaceutical preparations. Moreover, this work paves the way for NADES adoption in industrial application and formulation of ready-to-use extracts for food fortification.

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CRediT authorship contribution statement

Panić Manuela: Data curation, Formal analysis, Investigation, Methodology, Software, Writing - original draft. **Saša Drakula:** Data curation, Formal analysis, Investigation, Methodology, Software, Writing - original draft. **Giancarlo Cravotto:** Conceptualization, Funding acquisition, Supervision, Visualization, Writing - review & editing. **Robert Verpoorte:** Writing - review & editing. **Mirjana Hruškar:** Funding acquisition, Writing - review & editing. **Ivana Radojčić Redovniković:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft. **Kristina Radošević:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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