

Towards edible ionic liquids – Cholinium taurate

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Abstract: In this work, the possibilities and benefits of using an ionic liquid as a potential dietary supplement are presented and discussed for the first time. Ionic liquids prevent the development of microorganisms due to high ion concentration and thus, prevent perishability of the food products. Thermal stability, structure, as well as the experimental density and viscosity in the temperature range from 20 to 50 °C and at the atmospheric pressure 1×10^5 Pa of newly synthesized cholinium taurate ionic liquid, [Chol][Tau], are determined. According to the performed physicochemical characterization, it can be concluded that the synthesized ionic liquid is suitable for application in the food industry. The temperature variation of viscosity and density is discussed in terms of processes, packaging, and storage of [Chol][Tau]. Also, the antiproliferative activity of [Chol][Tau] is determined and compared with those obtained for ascorbic acid and Aspirin[®] as the standards.

Keywords: ionic liquids; food additive; synergistic effect; choline; taurine; anti-proliferative activity.

INTRODUCTION

In the last two decades, ionic liquids (ILs) have been explored and investigated due to their remarkable properties and potential applications in many industrial and technological areas. Because of their high chemical and thermal stability, the ability to dissolve large number of compounds and the fine tuning properties by the proper selection and the introduction of various functional groups in the IL components, they are now widely used in organic synthesis, catalysis, liquid–liquid extractions, gas absorption, dissolving of the cellulose, electrodeposition etc. Also, ionic liquids are very often attributed as “green solvents” mostly because of their low volatility and flammability.¹ This claim has

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launched a series of scientific research on their environmental impact, toxicity and biodegradability.² The results showed that the common and the most studied imidazolium and pyrrolidinium-based ionic liquids are not as benign as much as it was previously thought since they show significant toxicity to the cells and micro-organisms, as well as to plants and animals.³ The biodegradation of the IL is often not straightforward, since the product may be more toxic than the starting compound.⁴ As a consequence, the studies on the possible use of ionic liquids as a dietary supplement are almost non-existent, and ILs application in the food industry is mostly limited to use them as novel extractants in food analysis.^{5,6} On the other hand, there are numerous advantages of ionic liquids that may be applied in food industry such as good solubility in water, better bioavailability, colloidal particles dispersed in ILs⁷⁻⁹ and designed lipophilicity, which allows easier transport of the nutrients through the cell membrane and possibility of ILs synthesis with synergistic cation and anion performances.

Recent studies showed that tetraalkylammonium based ILs are less toxic than those based on the imidazolium, pyrrolidinium or pyridinium ions.² Also, the toxicity may be significantly decreased by introducing the polar hydroxyl, ether or carboxyl groups in the side alkyl chain of the cation.¹⁰ Therefore, the possibility of synthesis completely non-toxic ionic liquids, that could be used in the diet as the food additives, can be considered. One of the most promising ILs for that purpose are certainly those with choline based cation which contains quaternary ammonium group and the polar OH group in its structure, expecting thus very low toxicity.¹¹ Choline is biologically widespread micronutrient which cannot be completely degraded under aerobic conditions.¹² In recent years, many choline based ionic liquids were synthesized and characterized showing their low toxicity and excellent biodegradability.¹³⁻¹⁵ In the case of choline based ILs with amino acids as the anions, it was found that toxicity is even lower than the toxicity of the choline chloride, which is widely used as the food additive in the animal diet.¹²

Regarding the use of choline in the human diet, it was officially recognized by the US Institute of Medicine's Food and Nutrition Board in 1998, whereby the adequate daily intake for adult women is 425 mg (450 mg for pregnant women and 550 mg for lactating women) and 550 mg for adult men. Choline is synthesized in the liver *de novo* by the methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC), but these quantities are insufficient for the organism, and choline intake via food is necessary.¹⁶ In the body, choline is essential for the maintenance of structural integrity and signalling functions of the cell membrane, transport of the fats and cholesterol, as well as for the synthesis of the neurotransmitter acetylcholine. In particular, the most crucial role of choline is a donor of the methyl group, because as a precursor of betaine allows methylation of the homocysteine into methionine, and thus prevents hyperhomo-

cysteinemia, which is the cause of cardiovascular disease.^{17,18} Also, choline is essential for the brain development of the fetus and improves the visuomotor performance of healthy humans. Lack of choline causes liver and muscle damage.^{16,19} The usual daily dietary need is relatively easy to meet. Through food, choline comes in the form of lipid-soluble phosphatidylcholine (48 % of the total intake), and the most important sources are foods of animal origin, such as eggs, poultry, beef, pork, ham, and salmon. Another possibility of intake is the free water-soluble choline (23 %), and in that form can be found in the bread (wheat or rice), milk, bananas, orange juice and peanut butter. However, the research performed on athletes has shown that the free choline concentration decrease during intense exercise and that the supplementation of choline can improve endurance,^{20,21} and, therefore, choline may be found as an ingredient of many pre-workout sport supplements.^{22,23} In these products, choline is mainly found in the form of a cholinium tartrate and less frequently as cholinium citrate. It is known that most of the tartrate is destroyed in the intestinal tract by the present microorganisms,²⁴ while for the citrate is not found any positive effect on athletic performance or body composition.^{25,26} Thus, it is justified to replace them with the new non-toxic forms of choline-based salts with anions that exhibit an ergogenic effect. Also, the salts in the liquid forms such as ionic liquids can be easily combined with other components in the process of commercial production of dietary and sports supplements, as well as in the food industry in order to increase the nutritional value.

Therefore, in this work, new choline based ionic liquid with taurate as the anion is synthesized. Taurine is one of the sulfur-containing amino acids, does not form proteins and in natural form can be found in high concentrations in skeletal and cardiac muscle and brain tissue, making about 0.1 % of total human body weight. Numerous studies have confirmed the positive effect of taurine on sports performances,²⁷⁻²⁹ it decreases oxidative stress,³⁰ but also it shows an essential effect in the protection of the skeletal muscle and heart.³¹ Consequently, taurine is often added as an active component of energy drinks and other sports supplements.³²

For the newly synthesized cholinium taurate ionic liquid, the toxicity test is performed together with the physicochemical and thermal properties study in order to fully characterize it and to discuss the potential application of [Chol][Tau] as a novel additive to sports supplements. One of the goals is to evaluate cytotoxicity through determining cell growth effects of the newly synthesized ionic liquid, starting compounds of the synthesis (cholinium chloride and taurine) and standards (ascorbic acid and Aspirin[®]) in human fetal lung cell line MRC-5 derived from the healthy tissue.³³ Multi-endpoint bioassays that are based on the whole-cell response in mammalian cell lines are powerful indicators of metabolic, biochemical, and genetic alterations that arise under the influence

of evaluated compounds. They possess predictive power in terms of the risk to higher organisms.

EXPERIMENTAL

Synthesis of [Chol][Tau]

The calculated amount of taurine (Sigma Aldrich, $\omega \geq 99\%$) is dissolved in water at room temperature, and the obtained solution is titrated by the potentiometric method using the aqueous choline hydroxide (Sigma Aldrich, $\omega \geq 46\%$) in the concentration of $1.7524 \text{ mol}\cdot\text{dm}^{-3}$. After achieving equivalence point (pH 9.02), the obtained cholinium taurate (Fig. 1) is purified. Water is removed by the vacuum evaporation for 120 min at $70\text{ }^\circ\text{C}$ and the obtained [Chol][Tau] was kept in the vacuum desiccator over P_2O_5 . After drying, water content in the IL was found to be 215 ppm using the Karl Fisher titration.

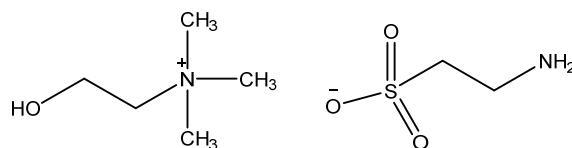


Fig. 1. Structure of [Chol][Tau] ionic liquid.

The structure is confirmed by ^1H -, ^{13}C -NMR and FTIR spectroscopy. The thermal stability is checked applying thermogravimetric (TG) and differential scanning calorimetry (DSC) analysis. Physicochemical properties such as density and viscosity are measured in the temperature range from 20 to $50\text{ }^\circ\text{C}$.

NMR spectra were recorded in D_2O at $25\text{ }^\circ\text{C}$ on a Bruker Advance III 400 MHz spectrometer. Tetramethylsilane was used as an accepted internal standard for calibrating chemical shift for ^1H and ^{13}C .

FTIR measurements were performed using a Thermo-Nicolet Nexus 670 spectrometer, equipped with a universal ATR sampling accessory. The measurements were performed with a total of 60 scans, at $25\text{ }^\circ\text{C}$, and a spectral resolution of 2 cm^{-1} in a range of ν from 750 to 4000 cm^{-1} . The software package Omnic, version 6.2, was used in the data acquisition and spectral analysis.

The thermal characterization of the sample was performed by thermogravimetric analysis using simultaneous TG/DSC thermal analyzer SDT Q600 (TA Instruments, USA). Sample ($\approx 2.5\text{ mg}$) was placed in an open platinum pan. Measurements were carried out in the nitrogen atmosphere at a flow rate of $100\text{ cm}^3\cdot\text{min}^{-1}$ up to $500\text{ }^\circ\text{C}$ with a heating rate of $10\text{ }^\circ\text{C}\cdot\text{min}^{-1}$. The instrument was calibrated (temperature and enthalpy) using the indium standard.

Density and viscosity measurements

The vibrating tube densimeter, Rudolph Research Analytical DDM 2911, with automatic viscosity correction, was used for density measurements. The accuracy and precision of the densimeter were $\pm 0.00005\text{ g}\cdot\text{cm}^{-3}$. The instrument was automatically thermostatted (Peltier type) within $\pm 0.01\text{ }^\circ\text{C}$ with automatic correction of the viscosity and was calibrated at the atmospheric pressure before each series of measurements. The calibration was performed using ambient air and bi-distilled ultrapure water in the temperature range from 20 to $50\text{ }^\circ\text{C}$.

Viscosity was measured using a Brookfield viscosimeter DV II+ Pro connected to the thermostat and filled with about 8 cm^3 of tested binary mixtures and pure components. The

spindle type (SC4-18) was immersed and the rounds per minute (*RPM*) was set in order to obtain a suitable torque. A viscometer cell protected from moisture with the compartment made by the manufacturer was calibrated using the liquids of different viscosities obtained from the manufacturer. Measurements were performed in the temperature range from 20 to 50 °C with the rotation speed of 60*RPM* for pure ionic liquid. The viscosity data at the corresponding temperatures were recorded automatically on a computer and then processed in Origin 2016.

Cell lines and cytotoxicity tests

Sulforhodamine B and antibiotic/antimycotic solution were purchased from Sigma Aldrich, fetal bovine serum and Dulbecco's modified essential medium were supplied from PAA Laboratories GmbH, trypsin was from Serva, and ethylenediaminetetraacetic acid (EDTA) from Laphoma. All substances were diluted in 9 mg·cm⁻³ NaCl solution and sterilized using 0.22 µm syringe filters. Ionic liquid and standards of cholinium chloride and taurine were studied in the concentration range from 125 to 2000 µg·cm⁻³, while ascorbic acid and Aspirin® were investigated in the range from 62.5 to 1000 µg·cm⁻³.

Cell growth activity was evaluated *in vitro* in human fetal lung cell line MRC-5 (ECACC 84101801). Cells were grown in Dulbecco's modified essential medium with 45 mg·cm⁻³ glucose supplemented with 10 % heat-inactivated fetal bovine serum (Biowest, Nuaille, France), 100 µg·cm⁻³ of penicillin, 100 µg·cm⁻³ of streptomycin and 0.25 µg·cm⁻³ of amphotericin B (antibiotic/antimycotic solution; Sigma-Aldrich). Cells were cultured in 25 cm³ flasks at 37 °C in the atmosphere of 5 % CO₂ and high humidity, and sub-cultured twice a week. A single cell suspension was obtained using 0.1 % trypsin with 0.04 % EDTA.

The cell lines were harvested and plated into 96-well microtiter plates at a seeding density of 4×10³ cells/well in a volume of 180 µL, and pre-incubated in complete medium supplemented with 5 % FBS at 37 °C for 24 h. Serial two-fold dilutions (20 µL) of tested substances were added to achieve final concentrations. An equal volume of solvent was added in control wells. After the addition of dilutions, microplates were incubated at 37 °C for 48 h. The cell growth was evaluated by colorimetric sulforhodamine B assay of Skehan *et al.*³⁴ Colour development was measured using Multiscan Ascent photometer at 540 nm against 620 nm as background.

The effect on cell growth was calculated as 100*AT/AC* (%), where *AT* is the absorbance of the test sample and *AC* of the control. Dose-effect (concentration-cell growth) curves were plotted for each treatment, and *IC*₅₀ values were determined. The results of cell growth activity were obtained in two independent experiments, each performed in quadruplicate (*n* = 8).

RESULTS AND DISCUSSION

Spectroscopy analysis

Obtained ¹H-NMR, ¹³C-NMR and FTIR spectra of [Chol][Tau] are presented in Figs. S1 and S2 of the Supplementary material of this paper.

¹H-NMR spectrum (D₂O, δ / ppm): 3.09 (*m*, 4H, NH₂CH₂CH₂SO₃); 3.28 (*bs*, 9H, N(CH₃)₃); 3.60 (*t*, 2H, CH₂N(CH₃)₃); 4.13 (*m*, 2H, CH₂OH).

¹³C-NMR spectrum (D₂O, δ / ppm): 36.65 (CH₂NH₂); 53.32 (CH₂SO₃⁻); 53.87, 53.90, 53.93 (N(CH₃)₃), 55.60 (CH₂OH); 67.44 (CH₂N(CH₃)₃).

FTIR spectrum of [Chol][Tau] is recorded in the wavelength range from 500 to 4000 cm⁻¹. IR (neat) 3348 (stretching OH); 3034 (sym. stretching CH,

($\text{N}(\text{CH}_3)_3$); 1603 (asym. bending NH_2); 1480 (rocking CH_3); 1360 and 1284 (wagging CH_2); 1170 (asym. stretching SO_3); 1086 (twisted NH_2 and twisted CH_2); 1031 (sym. stretching SO_3); 952 (asym. stretching CCO); 794 (stretching C–S); 605 (out-of-plane deformation γ SO_3); 581 (out-of-plane deformation γ SO_3); 520 (in-plane deformation γ SO_3).

Thermal analysis of [Chol][Tau]

In order to determine the thermal stability of the ionic liquid [Chol][Tau], the thermogravimetric analysis was performed. The results of thermal stability for potential food additive are very important, since they indicate which temperature range is suitable for the thermal processing of foods which contains the additive. The obtained thermogravimetric curve is presented in Fig. 2.

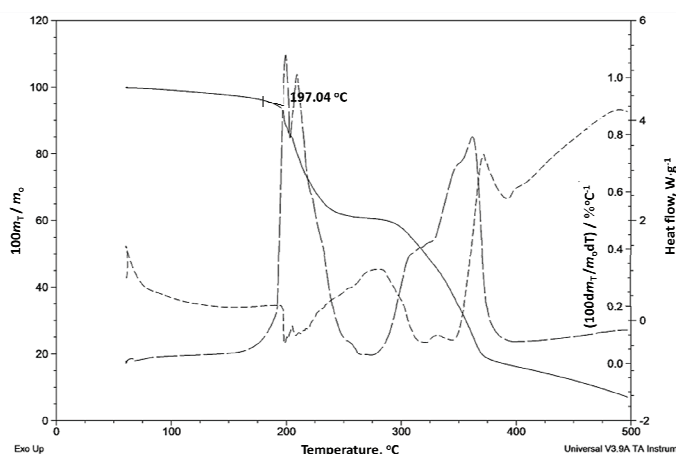


Fig. 2. TG (solid line), DSC (short dash) and DTA (long dash) analysis of [Chol][Tau], m_T – mass at temperature T , m_0 – initial mass.

It can be seen from Fig. 2 that the decomposition is a two-stage process, and begins at the 197 °C. Thermo-gravimetric analysis for choline ionic liquids with amino acids containing the carboxyl group instead of sulfonic group showed one-stage thermal decomposition¹³ between 150–203 °C. Based on the weight variation curves, the temperature by 2 and 5 % weight loss (T_2 %, T_5 %) was specified, and obtained values are 137.4 and 189.2 °C. The high thermal stability of ionic liquid does not require special temperature conditions during warehousing and preservation. Also, the presented thermal stability enables high-temperature technological processes during the food preparation. Thermal stability of [Chol][Tau] is lower in comparison with choline chloride³⁵ (300 °C), which is a consequence of weaker interactions between ions in ionic liquid, that originate from the more voluminous and better steric hindrance of taurate anion compared to chloride. On the other hand, higher thermal stability for taurine (328 °C)³⁶ can be explained by

his zwitterionic structure with strong electrostatic interactions and hydrogen bond formation in crystalline form,³⁷ while in ionic liquid taurine exists in an anionic form with strong repulsive electrostatic interactions.

Viscosity and density of [Chol][Tau]

Essential features of liquid food additives in many areas of food processing are viscosity and density. Knowing the physicochemical properties, with particular emphasis on viscosity and its variation with temperature, plays a vital role in design engineering processes in the food industry. The viscosity of food additives must be as low as possible for easier technical and technological processes and transport through the plant, reducing thus the price of operating costs.

Viscosity is closely associated with the textural and sensory attributes of the food product, such as appearance and flavour of food ingredients. Based on the viscosity measurements for a given product the sensory assessment of food texture could be predicted and may be changed in order to produce an additive with textural parameters acceptable to the consumer.

Viscosity results obtained from the experimental measurements are shown in Table S-I of the Supplementary material and graphically presented in Fig. 3. From Fig. 3 can be seen that the viscosity of [Chol][Tau] decreases with increasing temperature as expected.

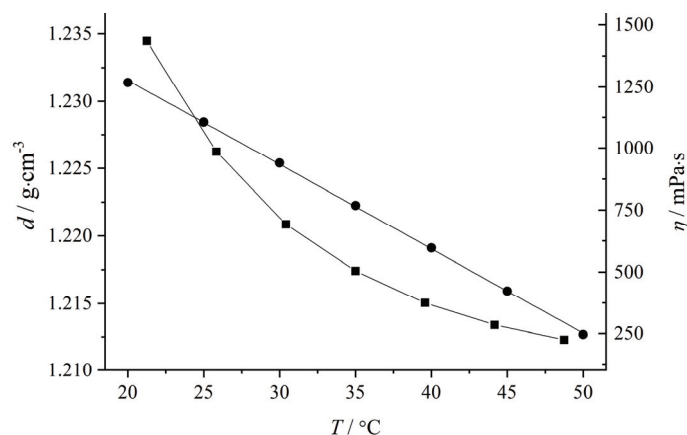


Fig. 3. Temperature dependence of [Chol][Tau] density (d , square) and dynamic viscosity (η , circle).

The obtained viscosity results for [Chol][Tau] are compared with commonly used food ingredients and food additives (Fig. 4). It is evident that the food industry uses even more viscous components than [Chol][Tau], which indicates that [Chol][Tau] ionic liquid is a promising ingredient and food supplement.³⁸

In Fig. 5 are presented and compared the changes of viscosity with temperature for [Chol][Tau] ionic liquid and honey as the natural representative mix-

ture of covalent compounds (glucose and fructose). Fig. 5 shows that [Chol][Tau] has about ten times lower viscosity in comparison with honey, while the temperature dependence of viscosity has a similar trend.

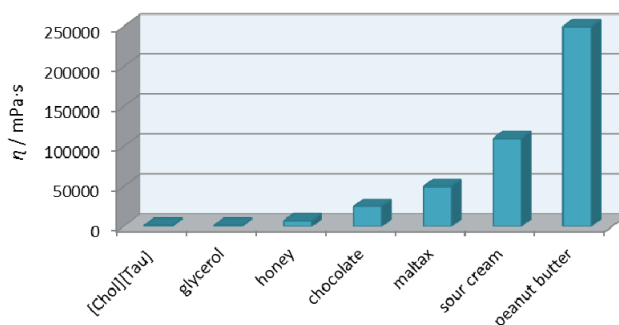


Fig. 4. Comparison of [Chol][Tau] viscosity at 25 °C with commonly used food ingredients and food additives.

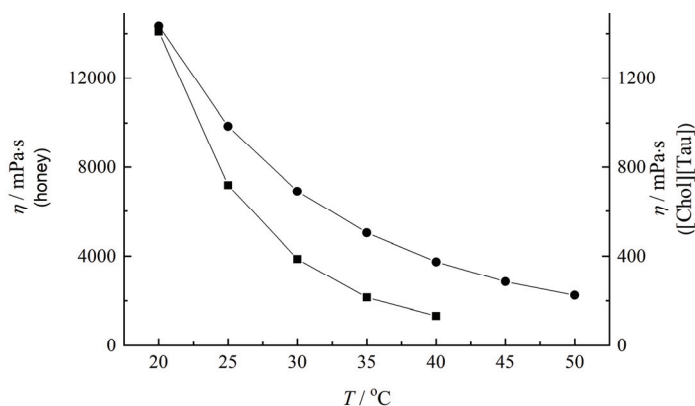


Fig. 5. Comparison of dynamic viscosity (η) for [Chol][Tau] (circle) and honey (square).

Variation of viscosity with temperature was fitted using the logarithmic form of the Arrhenius equation³⁸ (Fig. 6):

$$\ln \eta = C + E_a/RT \quad (1)$$

where C is the pre-exponential coefficient, E_a is the activation energy of viscous flow, and R is the universal gas constant. The activation energy of viscous flow was calculated from the slope of the Arrhenius plot and the obtained values amount to 48.83 and 90.85 $\text{kJ}\cdot\text{mol}^{-1}$ for [Chol][Tau] and honey, respectively, indicating the easier viscous flow of ionic liquid.

For the food manufacturing process optimization, the warehousing in cans and jars of foods being sterilized is partly dependent on their density.³⁹ Density is an important physicochemical property, and its temperature dependence is related to volume changes.

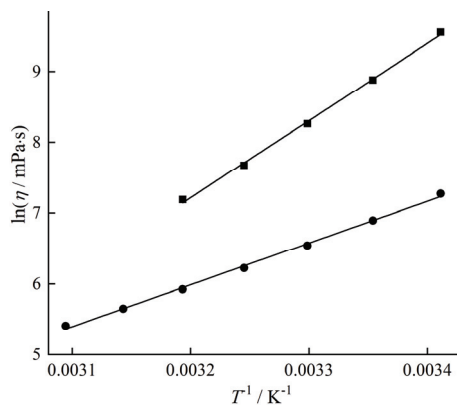


Fig. 6. Variation of $\ln \eta$ viscosity with T^{-1} for [Chol][Tau] (circle) and honey (square).

The measured density values are also shown in Table S-I and Fig. 3. The temperature dependence of [Chol][Tau] density is compared with those obtained for honey and presented in Fig. 7. It is evident that the value of [Chol][Tau] density decreases with the increase of temperature, wherein the density variation with temperature is less pronounced in the case of ionic liquid, in comparison with honey. This trend is a consequence of the ionic liquid specific structure and electrostatic interactions between cation and anion in [Chol][Tau]. A relatively small variation of ionic liquid density (and volume) with temperature, combined with negligible vapour pressure, makes it suitable for safe storage in sealed containers and non-temperature controlled conditions.

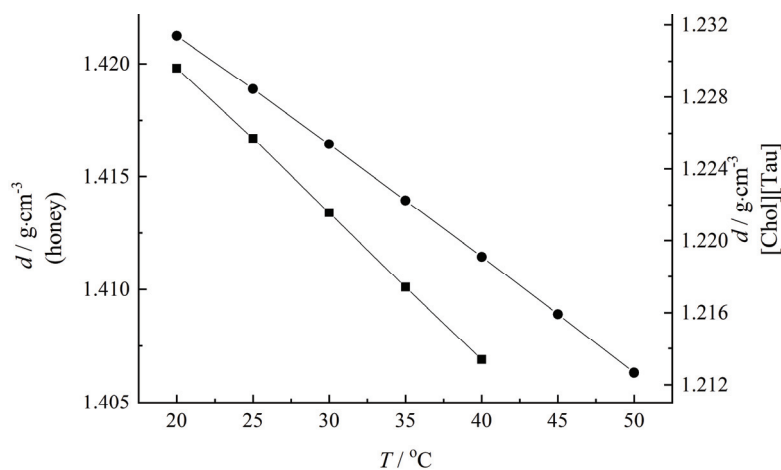


Fig. 7. Variation of density (d) with temperature for [Chol][Tau] (circle) and honey (square).

On the basis of the experimental densities, the thermal expansion coefficients for the of [Chol][Tau] and honey, α_p , can also be calculated:

$$\alpha_p = -\frac{1}{d} \left(\frac{\partial d}{\partial T} \right)_{p,m} \quad (2)$$

The determined thermal expansion coefficient tabulated in Table S-I and presented in Fig. 8.

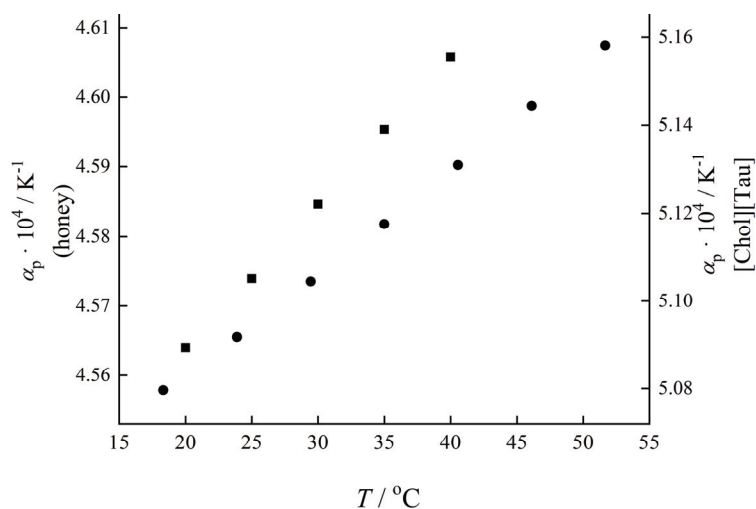


Fig. 8. Variation of thermal expansion coefficients with temperature for [Chol][Tau] (circle) and honey (square).

Based on these results, it is clear that the volume of ionic liquid slowly expands with an increase of the temperature, which facilitates the process of packaging and storage of [Chol][Tau] without the risk of sudden expansion in high-temperature storage conditions.

Cytotoxicity of [Chol][Tau]

In order to consider the potential application of [Chol][Tau] in the food industry, it is necessary to perform and evaluate the cytotoxicity of the synthesized ionic liquid. Antiproliferative activity of [Chol][Tau] was tested on the human non-tumor cell line (normal fetal lung fibroblasts MRC-5), and the obtained results are shown in Table I. MRC-5 is the well characterized human diploid fibroblast (HDF) cell line, derived from the healthy fetal lung tissue that is remarkably stable and retains predominantly normal diploid karyotype of the original tissue. Beside cytotoxicity of [Chol][Tau], the influence of commercial used substances (Aspirin[®], vitamin C) on cytotoxicity of MRC-5 cell line were examined and the obtained results are presented in Table I. The antiproliferative activity was expressed as IC_{50} value, defined as the dose of a compound that inhibits cell growth by 50 % and represent the effectiveness of a substance in inhibiting a

specific biological or biochemical function. According to well-known criteria, substances with IC_{50} values lower than $20 \mu\text{g}\cdot\text{cm}^{-3}$ had satisfying antiproliferative activity. Compounds with IC_{50} in the interval of $20\text{--}100 \mu\text{g}\cdot\text{cm}^{-3}$ can be considered weakly cytotoxic, and substances can be considered inactive if their IC_{50} values are higher than $100 \mu\text{g}\cdot\text{cm}^{-3}$.

Table I. Cell growth activity of ionic liquids and standards in MRC-5 cell line after 48 h exposition

Compound	$IC_{50} / \mu\text{g}\cdot\text{cm}^{-3}$
[Chol][Tau]	1426.78 ± 10.83^a
Choline chloride	$>2000^a$
Taurine	$>2000^a$
Ascorbic acid	398.60 ± 24.96^b
Aspirin [®]	$>1000^b$

^a125 to 2000 $\mu\text{g}\cdot\text{cm}^{-3}$ concentration range; ^b62.5 to 1000 $\mu\text{g}\cdot\text{cm}^{-3}$ concentration range

As can be seen from Table I, the starting compounds choline chloride and taurine can be considered as non-toxic in the investigated concentration range. On the other hand, ascorbic acid shows much higher cytotoxicity in comparison with [Chol][Tau].

CONCLUSION

Based on the performed physicochemical characterization, it can be concluded that the synthesized ionic liquid is suitable for application as a food additive. The obtained experimental results indicate that the volume of ionic liquid slowly expands with temperature, which can make the processes of packaging and storage of [Chol][Tau] easier and without the risk of sudden expansion in high-temperature storage conditions. In combination with negligible vapour pressure and relatively low viscosity of [Chol][Tau], manipulation and handling during the industrial processes and storage are less complicated. According to the examination of antiproliferative activity, [Chol][Tau] is less toxic than vitamin C.

SUPPLEMENTARY MATERIAL

Additional data are available electronically from the journal web site: <http://www.shd.org.rs/JSCS/>, or from the corresponding author on request.

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ИЗВОД

ХОЛИНИЈУМ-ТАУРАТ – ПУТ КА ЈЕСТИВИМ ЈОНСКИМ ТЕЧНОСТИМА

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У овом раду су је предочена могућност употребе јонских течности као додатака у исхрани људи, по први пут. Јонске течности спречавају развој микроорганизама у прехранбеним производима, услед могућности постизања високих концентрација електролита у њима. Испитана је термичка стабилност, структура и измерене вискозности и густине у температурном опсегу 20 до 50 °C и на атмосферском притиску (1×10^5 Pa) синтетисане јонске течности – холинијум-таурата. На основу резултата физичко-хемијских мерења, може се закључити да је испитивана јонска течност погодна за манипулацију у процесима прехранбене индустрије. Испитана је и антипролиферативна активност синтетисане јонске течности, а резултати су упоређени са стандардним стандардним супстанцама – аспирином и витамином Ц.

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