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Vibrational spectra and antimicrobial activity of selected bivalent cation benzoates

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ABSTRACT

Selected bands of FT-IR spectra of Mg(II), Ca(II), Cu(II) and Zn(II) benzoates of both solid state and water solution, were assigned to appropriate molecular vibrations. Next evaluation of electronic charge distribution in both carboxylic anion and aromatic ring of studied compounds was performed. Classical plate tests and turbidimetry measurements, monitoring growth of bacteria *Escherichia coli, Bacillus subtilis* and yeasts *Pichia anomala* and *Saccharomyces cerevisiae* during 24 h of incubation, in optimal growth conditions (control) and in medium with addition of studied benzoate (concentration of 0.01% expressed as the concentration of benzoic acid), proved antimicrobial activity of studied compounds against investigated micro-organisms.

PLS (partially least square) and PCR (principal component regression) techniques were applied to build a model, correlating spectral data reflecting molecular structure of studied compounds, with degree of influence of those compounds on growth of studied micro-organisms. Statistically significant correlation within cross validation diagnostic of PLS-1 calibration was found, when log 1/T of selected spectral regions of water solution samples were used as input data. The correlation coefficients between predicted with PLS calibration based on created 1, 2 or 3 factor models, and actual values of antimicrobial activity were: 0.70; 0.76, 0.81 for *P. anomala, B. subtilis*, and *E. coli*, respectively. Log(PRESS) values of appropriate models were 2.10, 2,39 and 3.23 for *P. anomala, B. subtilis*, and *E. coli*, respectively.

1. Introduction

Benzoic acid derivatives, e.g. salicylic, nicotinic, picolinic and *o*-, *m*-, *p*-halogenobenzoic acids and their complexes with different metal cations were investigated in our previous papers [1–5]. Qualitative and quantitative influence of metal cations on the electronic charge distribution in ligands which are of importance for various industrial branches (e.g. pharmacy, cosmetics, food) was established. Metal parameters, such as ionic potential, atomic mass and electronegativity were proved to be factors responsible for the effects of various metal cations on the electronic structure of carboxylate anion and aromatic ring of various benzoic acid derivatives. The main conclusions of our previous studies were drawn based on analysis of spectral data obtained with use of different methods (IR and Raman, UV–vis, ^{1}H and ^{13}C NMR). We have also tested survival of micro-organisms in presence of studied benzoic acid derivatives to evaluate their antimicrobial potential [6–9].

As the vibrational spectra are complicated due to a number of overlapping spectral bands, the techniques of PCA (principal component analysis), PCR (principal component regression) and PLS (partially least square) are commonly applied to reduce large number of experimental data. Those statistical procedures are well known as a proper and valuable tool to simplify IR spectral data, and therefore have been successfully used by many authors [10–13]. PLS is particularly useful when the number of predictors is higher than number of observations and ordinary least squares regression either fails or produces small coefficients with large standard errors.

It is well known that antimicrobial activity or more general biological activity of chemical compound varies significantly due to even minimal changes in chemical structure. One clear example is antitumor active *cis*-platine with its trans isomer lacking antitumor properties [14]. Number of authors determine SAR (structure–activity relation), considering various parameters of studied compounds. One of the parameters is π value, defined as difference between $\log P_X$ and $\log P_H$, where P_X is the partition coefficient of benzoic acid in the octanol–water system, and P_H is the partition coefficient of a given compound in the same system [15]. Also wavenumber of simple NO stretches of series of compounds containing NO group is considering as parameter responsible for antimicrobial activity [16]. The theory of SAR lists a lot of different parameters (e.g. atomic mass, electronegativity, dipole momentum, hydrogen bonds, atomic formal charge, HOMO/LUMO, intermolecular interaction) determining biological activity [17]. Most of listed parameters are undoubtedly related to molecular

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structure of a given compound. In fact molecular structure and electronic charge distribution determine micro and macro properties, including biological activity. The task is to construct proper mathematical formula connecting features of both structure and activity. In our study [6–8], as well as in study of other authors [16], data provided by IR spectra are considered as extremely important, as they include information on movement of atoms and amount of bonds energy. Latter features are in turn responsible for molecular structure (e.g. bond length or angels).

In our previous studies we have proved the relation between IR spectral data and antimicrobial activity of some benzoic acid derivatives. Continuing our research in this paper we propose PLS technique to correlate IR data with antimicrobial properties of selected bivalent cation benzoates. Our investigation is aimed at finding robust model to establish correlation between molecular structure characterised by spectral data reduced with PCA or PLS, and antimicrobial activity of series of compounds, which is quite new idea.

In this paper we have studied bivalent cation benzoates, namely Mg(II), Ca(II), Cu(II) and Zn(II) benzoates to compare their electronic structures characterised by IR data, evaluate antimicrobial activity against two bacteria and two yeast species, and finally test the existence of the structure–antimicrobial activity relation. Proposed compounds are water-soluble (especially Mg and Ca are of good water solubility), which is an advantage in their potential practical application. Moreover, studied metals are of great interest from the nutritional point of view [18,19] which makes studied salts particularly interesting due to potential application as food preservatives. Although there are spectral data of herein proposed complexes in literature [20–22], there is a lack of in-depth discussion on the structure–antimicrobial activity relation based on spectral data of water solution samples. Moreover, spectra assigned and discussed before [20,21] by Lewandowski, were registered with an older type of apparatus. To achieve the goals of this paper and to improve spectra quality (e.g. bands wavenumber or under band area), we registered spectra with use of FT-IR technique.

2. Materials and methods

The benzoic acid was Sigma analytical reagent. The complexes of Ca were prepared by dissolving benzoic acid in aqueous solution of metal hydroxide (Merck analytical reagent) in a stoichiometric ratio. Solid benzoates of the studied metals were obtained by mixing aqueous solutions of soluble salts of the metals with sodium benzoate. For instance, zinc benzoate was synthesized by mixing equal volumes of solutions of $ZnCl_2$ ($0.25\ \mathrm{mol\,dm^{-3}}$) with C_6H_5COONa ($0.5\ \mathrm{mol} \times \mathrm{dm^{-3}}$).The precipitates were recrystallized, filtered off, washed with water, and dried first at room temperature and then at 95 °C. Benzoates obtained under the above conditions are anhydrous. Analytical data for the compounds were complete in accordance with the expected metal-to-benzoate stoichiometries, i.e., 1:2 for Mg, Ca, Cu and Zn.

KBr pellets were used for transmittance of IR measurements of studied benzoates within the range of MIR (400–4000 $\mathrm{cm^{-1}}$) with a Perkin Elmer System 2000 FT-IR spectrophotometer. The resolution was 2 $\mathrm{cm^{-1}}$. For each studied compound, five separated pellets were prepared to average influence of pellet preparation (grinding and pressing with KBr) on the wavenumbers of spectral bands – possibility of such influence was discussed in our previous paper [7]. Moreover, for magnesium benzoate HATR technique, in which pure sample (without KBr matrix) is used, was applied to confirm wavenumbers of spectral bands discussed in this paper. Pellets were prepared by mixing KBr powder (Sigma reagent) with powdered benzoate, dried during 10 h in the temperature of 90 °C, in ratio of 300 mg:1 mg. Powder mixture was grounded in the ball mill for 2 min. Then powder was pressed in the hydraulic press (Perkin Elmer) with 10 T of pressure for 1 min. Transparent pellets were placed in the holder and spectra were registered against background spectrum. Background spectrum was registered with pellet prepared in the same way as described above, but with KBr powder alone.

Two different techniques: attenuated total reflectance and transmittance were used to register water solution spectra. Reflectance technique spectra were registered using Perkin Elmer HATR accessory (ZnSe, 60° crystal), while transmittance spectra were registered in film, using KRS plates. In both cases System 2000 Perkin Elmer spectrophotometer was used, however spectral range was 2000–600 and 2000–400 $\mathrm{cm^{-1}}$ for reflectance and transmittance techniques, respectively. Two techniques had to be applied as intensities of some bands were extremely low when using reflectance technique alone.

Two species of bacteria: *E. coli*, and *B. subtilis* as well as two species of yeasts: *S. cerevisiae* and *P. anomala* were used for antimicrobial tests. (*S. cerevisiae* – ATCC 9763, *E. coli* – ATCC 25922, *B. subtilis* – ATCC 6633, *P. anomala* – the collection of Institute of Agricultural and Food Biotechnology in Warsaw). The studied micro-organisms were inoculated on broth medium and stored at 35 °C, for 24 h. The growth of the tested cells was standardized using turbidimetry analysis by measuring optical density at 600 nm for *B. subtilis, E. coli, P. anomala*, and *S. cerevisiae* with a V-2001 HITACHI spectrophotometer [23]. The microbiological tests of the studied compounds (the final concentration was 0.01% expressed as the concentration of benzoic acid) were carried out in water medium. The ultra pure water was used (deionised water, resistance 18.2 MΩ/cm). The samples were incubated at 35 °C for bacteria and at 25 °C for yeast. The increase in the number of colonies was estimated after 24 h of incubation. The results were presented as the percentage of growth inhibition compared to the control sample (the optimal conditions for growth of micro-organisms).

$$\%\ \text{of growth inhibition} = 100\% - [(A_1 \times 100\%)/A_0]$$

A_1, the value of optical density in the sample with the tested compound; A_0, the value of optical density in the control sample.

Statistical calculations were done using STATGRAPHICS Plus 2.1 package and GRAMS/AI software with PLS/IQ extension running on Windows XP platform. Cross validation diagnostic in PLS-1 calibration was used to create the robust statistical model. Maximal number of factors was set to 3. One file was left out while building the model with cross validation diagnostic. Two spectral regions 1571–1528 and 1415–1366 $\mathrm{cm^{-1}}$ (94 points altogether) out of several tested regions were set, to achieve the best correlation with data on antimicrobial activity. No one of studied compounds was outlier.

Log 1/T values against wavenumbers of Na, Mg, Ca, Cu and Zn benzoates were measured. Correlation coefficients (R^2) and log(PRESS) (Prediction Residual Error Sum of Squares) values were used to evaluate and select best models.

3. Results and discussion

3.1. Spectral data

Wavenumbers of selected spectral bands of solid state samples gathered in Table 1, were assigned based on our previous experience [24] and data found in literature [25]. Presence of some characteristic bands (i.e. ν(COO^-), β(COO^-)), or disappearance of some other bands (i.e. ν(O–H), β(O–H) and γ(O–H)) in spectra of benzoates in comparison to spectrum of benzoic acid proves that given metal replaces the carboxylic group hydrogen.

Bands present in the spectra originate from both carboxylic anion vibrations and ring vibrations. Bands originating from carbox-

Table 1
Wavenumbers, intensities and assignments of selected bands occurring in the IR spectra of Ca(II), Mg(II) Cu(II) and Zn(II) benzoates

Mg benzoate		Ca benzoate		Cu benzoate		Zn benzoate		Assignment
Wavenumber	Intensity	Wavenumber	Intensity	Wavenumber	Intensity	Wavenumber	Intensity	
1631	vs	1630	vs	1633	vs	1639	vs	$\nu(\mathrm{C}\overset{\cdots}{-}\mathrm{C})_{\mathrm{ar}}$
1598	vs	1595	vs	1597	vs	1611	vs	$\nu(\mathrm{C}\overset{\cdots}{-}\mathrm{C})_{\mathrm{ar}}$
1553	vs	1558	vs	1555	vs	1575	vs	$\nu_{\mathrm{asym}}(\mathrm{COO}^-)$
1492	m	1498	m	1492	m	1493	m	$\nu(\mathrm{C}\overset{\cdots}{-}\mathrm{C})_{\mathrm{ar}}$
1423	vs	1444	vs	1412	vs	1382	vs	$\nu_{\mathrm{sym}}(\mathrm{COO}^-)$
1069	w	1071	m	1069	w	1071	m	β(C–H)
1029	w	1025	m	1027	m	1026	m	β(C–H)
930	w	935	w	931	w	941	w	β(C–H)
846	m	846	m	846	m	841	m	$\beta_{\mathrm{sym}}(\mathrm{COO}^-)$
767	w	767	w	767	w	772	m	$\gamma_{\mathrm{sym}}(\mathrm{COO}^-)$
524	w	521	w	520	w	572	w	$\beta_{\mathrm{asym}}(\mathrm{COO}^-)$

vs, very strong; s, strong; m, medium; w, weak; vw, very weak; sh, shoulder.

ylate anion vibrations are: (1) carboxylic anion asymmetric stretches $\nu_{\mathrm{asym}}(\mathrm{COO}^-)$, located at 1553, 1558, 1555 and 1557 cm^{-1} for Mg, Ca, Cu and Zn benzoates, respectively; (2) carboxylic anion symmetric stretches $\nu_{\mathrm{sym}}(\mathrm{COO}^-)$ at 1423, 1444, 1412, and 1382 cm^{-1} for Mg, Ca, Cu and Zn benzoates, respectively; (3) in plane symmetric deformations $\beta_{\mathrm{sym}}(\mathrm{COO}^-)$ at 846, 846, 846 and 841 cm^{-1} for Mg, Ca, Cu and Zn benzoates, respectively; (4) in plane asymmetric deformations $\beta_{\mathrm{asym}}(\mathrm{COO}^-)$ at 524, 521, 520 and 575 cm^{-1} for Mg, Ca, Cu and Zn benzoates, respectively. (5) out of plane symmetric deformations $\gamma_{\mathrm{sym}}(\mathrm{COO}^-)$ at 767, 767, 767 and 772 cm^{-1} for Mg, Ca, Cu and Zn benzoates, respectively. The remaining bands present in spectra originate from aromatic ring vibrations, the bands originating from metal–oxygen vibrations are below spectral range involved in present measurement.

The wavenumbers of asymmetric and symmetric stretches (both of very strong intensity) are highly sensitive to the electronic charge distribution, and therefore to the structure of carboxylate group. Consequently, the separation of those bands i.e. $\Delta = \nu_{\mathrm{asym}}(\mathrm{COO}^-) - \nu_{\mathrm{sym}}(\mathrm{COO}^-)$ is also strongly indicative of the structure of a given carboxylate. Δ is the parameter well known in literature [26,27]. A general decreasing trend of the following order: uncoordinated acid > unidentate coordination > bidentate (bridging > chelating) coordination > free carboxylate anion is observed for both stretching frequencies and/or band separation values in carboxylate metal complexes. Also the IR spectra of metal–acetate complexes are often described in terms of the value of wavenumber separation between the asymmetric and symmetric stretches Δ rather than absolute wavenumbers [28,29]. According to Deacon [28] and Nakamoto [29], very low Δ values (around 100 cm^{-1}) indicate chelation or a combination of chelation and bridging while larger Δ values (around 150 cm^{-1}) are indicative of chelating or bridging acetate coordination, while substantially greater value (around 200 cm^{-1}) are indicative of monodentate acetate coordination. The Δ values calculated from theoretical wavenumbers [30] support the empirical data. Large Δ values around 241 cm^{-1} are obtained from monodentate complexes, while significantly smaller values (around 127 cm^{-1}) are calculated for bidentate and bridging structures. Although Δ is commonly used and verifiable parameter, in order to distinguish between different carboxylic anion configurations additional information about the system under study is sometimes required (e.g. location of $\beta_{\mathrm{sym}}(\mathrm{COO}^-)$ band) [25]. Shift of the wavenumber of $\beta_{\mathrm{sym}}(\mathrm{COO}^-)$ band toward the lower wavenumbers is recognized as identification of more ionic structure of carboxylate anion. Following data in Table 1, for studied here complexes Δ values are as follows: 130, 114, 143, 193 for Mg, Ca, Cu and Zn benzoates, respectively, while wavenumbers of $\beta_{\mathrm{sym}}(\mathrm{COO}^-)$ are 846, 846, 846, 841 for Mg, Ca, Cu and Zn benzoates, respectively. Thus, $\beta_{\mathrm{sym}}(\mathrm{COO}^-)$ bands located at around 846 cm^{-1} each, do not really distinguish studied complexes, while Δ separation does. Regular increase in the Δ value in the following order: Mg, Ca, Cu, Zn indicates chelation structure in case of Mg benzoate, bridging structure in case of both Ca and Cu benzoates and monodentate structure in case of Zn carboxylates, which suggests coordination number of central ion (Zn^{2+}) equal to 2, similarly as in ordinary zinc salts. To recapitulate, the bonds within carboxylic anion of studied complexes are of different polarity. Consequently, structure of carboxylate anion significantly varies along with studied bivalent cation change.

Moreover, a different influence of a given metal cation on vibrations of aromatic ring is observed. The metal–aromatic ring interaction is transferred by carboxylate anion (M–O, C–O and C–C bonds). Consequently, bands originating from aromatic ring vibrations are located at different wavenumbers, depending on coordinated metal. In the high frequency spectral region, above 1600 cm^{-1}, two bands occur (see the first and second rows in Table 1) and both of them slightly distinguish studied complexes. Alike, other aromatic ring bands listed in rows seven and eight in Table 1, distinguish studied compounds as well. Wavenumbers of the bands originating from the same ring vibrations of studied compounds vary, which indicates that the strength and type of metal–ring interactions, transmitted through the differently structured carboxylic anions (differences between structures of carboxylic anions of the studied complexes were proved above) depend on the carboxylic anion structure.

Data of water solutions of studied compounds in concentration 0.01% are gathered in Table 2. Less number of bands occurs in spectra of water solution samples compared to spectra of solid state samples. Bands originating from carboxylic anion vibrations are of small intensity (contrary to the same bands in spectra of solid state samples), likely due to intermolecular $\mathrm{H_2O{-}COO^-}$ interaction. On the other hand, bands originating from ring vibrations are of strong intensity and wide shape. That was similar.

Spectra of water solution of different metals are analogous, thus depicted by the similar number of related intensity bands located in comparable spectral range. Very intense and wide band of 8a or 8b vibrations is located in the 1810–1430 cm^{-1} region. In the right shoulder of this intensive signal (around 1540 cm^{-1}), characteristic band of weak intensity, originating from asymmetric carboxylic anion stretches, appears. Then around 1380 cm^{-1} a band of medium intensity, originating from symmetric stretches of COO^- group, can be found. Within the spectral region of 1100–400 cm^{-1} two bands of very strong intensity are located around 770 and 470 cm^{-1}, respectively. They are present in spectra of all studied compounds. Those bands originate from ring vibrations

Table 2

Wavenumbers, intensities and assignments of selected bands occurring in the IR spectra of water solution of Ca(II), Mg(II) Cu(II) and Zn(II) benzoates

Mg benzoate		Ca benzoate		Cu benzoate		Zn benzoate		Assignment
Wavenumber	Intensity	Wavenumber	Intensity	Wavenumber	Intensity	Wavenumber	Intensity	
1644	vs	1637	vs	1620	vs	1645	vs	$\nu(\mathrm{C}{\cdots}\mathrm{C})_{ar}$
1559	sh	1548	sh	1538	sh	1546	sh	$\nu_{asym}(\mathrm{COO}^-)$
1389	vs	1390	m	1328	m	1392	s	${}^{\nu}_{sym}(\mathrm{COO}^-)$
1318	m	1307	vw	–		1319	vw	
–		1177	vw	–		1143	m	
1074	w	1029	w	1069	m	1120	v	$\beta(\mathrm{C–H})$
755	s	776	s	829	s	779	s	$\beta_{sym}(\mathrm{COO}^-)$
523	s	487	s	576	s	487	s	$\beta_{asym}(\mathrm{COO}^-)$

vs, very strong; s, strong; m, medium; w, weak; vw, very weak; sh, shoulder.

and overlap all other bands which are expected in this region (e.g. out of plane vibrations of carboxylic anion).

Resuming spectral data, it is to say that studied here salts of different metals differ significantly in both structure of carboxylate anion and aromatic ring. Therefore, it is likely that their antimicrobial activity would differ.

3.2. Biological data

Table 3 contains data on inhibition effect of the studied Mg, Ca, Cu and Zn benzoates on the growth of *E. coli, B. subtilis, P. anomala* and *S. cerevisiae* after 24 h of micro-organism incubation. Data gathered in Table 3 are averages of five replications. Averages in Table 3 are followed by confidence intervals. In addition to tests samples, control samples in conditions optimal for micro-organisms growth were incubated. The sodium benzoate, which is commonly used preservative agent, was used as a reference material, as it was done in our previous papers [7,8]. Sodium benzoate concentration in current experiments was higher than in the previous papers, as it had to correspond to the level of concentration of currently studied compounds. Although Na benzoate concentration was 2-fold higher in current experiments, in some cases its activity against the same micro-organisms studied currently and before, was unexpectedly lower comparing to previous results. This phenomenon might be explained by fact that some inhibitors demand specific concentration to show maximum activity, and increased concentration does not improve activity.

Some data presented in Table 3 are of relatively high confidence intervals (e.g. −11.2 ± 2.4 for Mg benzoate against *E. coli*). One must keep in mind, that those are data obtained from biological measurements, and although all experimental conditions were kept unchanged for each sample, high variations might have occurred. Activity of the studied compounds changes from growth inhibition to growth stimulation which is denoted by minus signs in Table 3. Stimulation effect, as well as inhibition effect, must be evaluated with great care, as this is the average of five independent measurements, performed in the same experimental conditions. In order to compare inhibition strength of studied compounds, it is necessary to take the confidence interval into account. For example, stimulation effect was observed for Mg benzoate action against *E. coli*. In this case, the result of each replicate was under zero, with confidence interval ±2.4 and average value of −11.2. Similar stimulation was observed for Mg or Ca benzoates action against *S. cerevisiae*. Action of Cu and Zn benzoates against *E. coli* and Mg benzoate against *B. subtilis* can be evaluated as neutral as in some experiments small inhibition while in others small stimulation was observed. On the other hand, Mg benzoate inhibited *P. anomala* growth in each replicate, Ca benzoate inhibited *E. coli, B. subtilis, P. anomala*, in every experiment and Cu and Zn benzoate inhibited *B. subtilis, P. anomala* and *S. cerevisiae* each time. In order to perform statistical correlations within this paper, the averages were set as the values of antimicrobial activity of studied compounds.

After 24 h of incubation the studied compounds influence the growth of studied micro-organisms except *P. anomala* at a significantly lower level than control sodium benzoate. Among studied benzoates, there are statistically significant differences between the level of activity, denoted in the Table 3 by "*p*" footnote, therefore it was possible to distinguish those compounds by the quantity of their action against micro-organisms. The level of growth inhibition increases in the following order Cu > Zn = Cu > Mg; Cu = Ca = Zn > Mg; Cu = Ca = Zn = Mg; and Zn > Cu > Mg = Ca for *E. coli, B. subtilis, P. anomala* and *S. cerevisiae*, respectively.

To evaluate the effect of cation alone on *E. coli* and *S. cerevisiae* survival, microbiological tests were done with water-soluble inorganic salts of Mg^{2+}–[$Mg(NO_3)_2$], Ca^{2+}–($CaCl_2$), Cu^{2+}–($CuSO_4$), and Zn^{2+}–($ZnCl_2$) in the concentration of given cation equal to the concentration of this cation in case of benzoate samples (0.01%). Results are presented in Table 4. Our assumption was that if the antimicrobial activity depends only (mainly) on cation, the inhibition degree expressed by inorganic salt built of the same cation as

Table 3

The degree of growth inhibition of bacteria *Escherichia coli* and *Bacillus subtilis* and yeast *Pichia anomala* and *Saccharomyces cerevisiae* caused by Mg, Ca, Cu, Zn and Na benzoates after 24 h of incubation[a]

		Escherichia coli	*Bacillus subtilis*	*Pichia anomala*	*Saccharomyces cerevisiae*
1	Na benzoate	20.5 ± 16.6 $p_{1-2,3,4,5} < 0.05$	22.1 ± 1.3 $p_{1-2,3,4,5} < 0.05$	23.4 ± 8.7	16.9 ± 6.6 $p_{1-2,3,4,5} < 0.05$
2	Mg benzoate	−11.2 ± 2.4 $p_{2-3,4,5} < 0.05$	0.1 ± 1.3 $p_{2-3,4,5} < 0.05$	23.2 ± 2.3	−2.4 ± 1.8 $p_{2\text{-}4,5} < 0.05$
3	Ca benzoate	2.7 ± 0.4 $p_{3-4,5} < 0.05$	6.0 ± 1.7	19.2 ± 8.8	−5.3 ± 3.0 $p_{3-4,5} < 0.05$
4	Cu benzoate	−1.4 ± 2.5	7.6 ± 2.1	24.7 ± 2.8	4.6 ± 3.1
5	Zn benzoate	−0.6 ± 2.6	5.3 ± 4.4	27.0 ± 5.4	7.6 ± 3.9

[a] The negative values stand for growth stimulation. Each value is the average of five replicates and is followed by its confidence interval. The concentration of each compound was 0.01% expressed as a concentration of benzoic acid. The *p* value stands for statistically significant difference between samples denoted by numbers from 1 to 5 at the 95% confidential level.

Table 4
The degree of growth inhibition of bacteria *Escherichia coli* and yeast *Saccharomyces cerevisiae* exhibited by Mg, Ca, Cu and Zn inorganic salts after 24 h of incubation

Inorganic salts	*S. cerevisiae*	*E. coli*
24 h of incubation		
Mg^{2+}	−9.4 ± 7	3.6 ± 6
Ca^{2+}	−12.0 ± 1	−2.1 ± 7
Cu^{2+}	−9.2 ± 7	3.8 ± 1
Zn^{2+}	8.0 ± 2	7.4 ± 2

in case of benzoate, will be the same as inhibition degree shown by studied benzoate. In the case of *E. coli* there is a statistically significant difference between the degree of action of Zn, Ca, Cu and Mg benzoates and their inorganic salts, while in the case of *S. cerevisiae* the differences are statistically insignificant for Zn salts.

A conclusion can be drawn that in case of benzoates anion is mainly responsible for antimicrobial action.

3.3. Structure–antimicrobial activity relationship

Chemometrics is widely used to establish the correlation between two sets of variables derived from the same samples. Within multivariate models IR spectral data are related with data of content of proteins, sugars, fats and other components of foodstuff [31–33] or with physical and chemical properties of gasoline [34]. This technique enables to achieve results similar to those obtainable with standard methods at shorter times, lower costs and safer sample handling. Relating wavenumbers of selected IR bands with antimicrobial activity is quite new idea. The model once validated can be applied as a tool in routine procedure, e.g. for preselecting particularly interesting compounds from large group of compounds due to biological activity.

In our approach different data were processed using simple regression followed by multivariate regression. Spectral parameters e.g. various single band wavenumbers, band intensities, or differences between selected band wavenumbers or non-spectral parameters e.g. atomic mass, reversed atomic mass, electro negativity were initially analyzed due to their relation to microbiological activity. Unfortunately, relation between those parameters and biological activity was at statistically insignificant level.

For solid state samples statistically significant models were only obtained when frequencies of various spectral bands were considered altogether. Data of solid state benzoate samples and data of activity against *E. coli* were initially processed with PCA technique. PCA however, produced principal components which did not really distinguish studied compounds towards their spectral structure, as their values were almost the same for every compound, which eventually resulted in extremely high (enormous) correlation coefficient. Contrary, PLS produced three-elemental model correlating spectral data and the antimicrobial activity against *E. coli* with *t* relatively high correlation coefficient equal to 0.83 and log(PRESS) = 3.12. Data of activity against remaining micro-organisms did not produced statistically significant models.

Spectral data of water solution samples differ significantly from data of solid samples (see Tables 1 and 2). Water solution data seem to be more suitable for building a statistical model, as micro-organism incubation was conducted in water environment. Various spectral regions were analyzed in order to find good correlation, while applying two of them (1571–1528 and 1415–1366 cm^{-1}) altogether within PLS-1 calibration produced high correlation coefficients and relatively low PRESS values. Table 5 presents results obtained from cross validation diagnostics used within PLS-1 calibration.

For three out of four studied micro-organisms statistically significant correlation between actual and predicted degree of growth inhibition was found. For *E. coli* (24 h of incubation) correlation coefficient equal to 0.81 and log(PRESS) = 3.23 was obtained with one-factor model. One factor model was selected, as R^2 for two- and three-factor models were significantly lower, while log(PRESS) significantly higher. For *B. subtilis* one-factor model produced correlation coefficient of 0.70, with log(PRESS) = 2.39, and for *P. anomala* three-factor model produced correlation coefficient of 0.76 with log(PRESS) = 2.10. Two- and three-factor models obtained for *B. subtilis* and one and two factor models obtained for *P. anomala* showed significantly lower R^2 and significantly higher log(PRESS). Fig. 1 presents plot of actual versus predicted antimicrobial activity of studied compounds against *B. subtilis*.

Table 5
Statistical data obtained with PLS-1 calibration (cross validation diagnostic with one file out)

	Number of factors in model	R^2	Log(PRESS)
PLS calibration			
E. coli	1	0.81	3,23
B. subtilis	1	0.70	2,39
P. anomala	3	0.76	2,10
S. cerevisiae	2	0.55	3,46

Two spectral regions of total 94 points of five different samples were involved in PLS-1 calibration.

Correlation coefficients found for *S. cerevisiae* with application of spectral data of water solution samples and cross validation diagnostic technique were statistically less significant (see Table 5).

Statistically significant correlation coefficients obtained for studied micro-organisms and compounds confirm the existence of correlation between IR wavenumbers and biological activity. Even small changes in chemical structure change biological properties, and all those changes are reflected in IR spectra. Spectral regions involved in the model created contain stretches of symmetric and asymmetric vibrations – of COO^- group as well as aromatic ring vibrations. Wavenumbers of bands of anion or bands of ring analyzed separately according to Lambert–Beer law did not produce good results. So that, whole molecule except cation is responsible for biological activity of studied series of benzoates. Cation insignificance is confirmed by lack of good correlation in case of solid state samples.

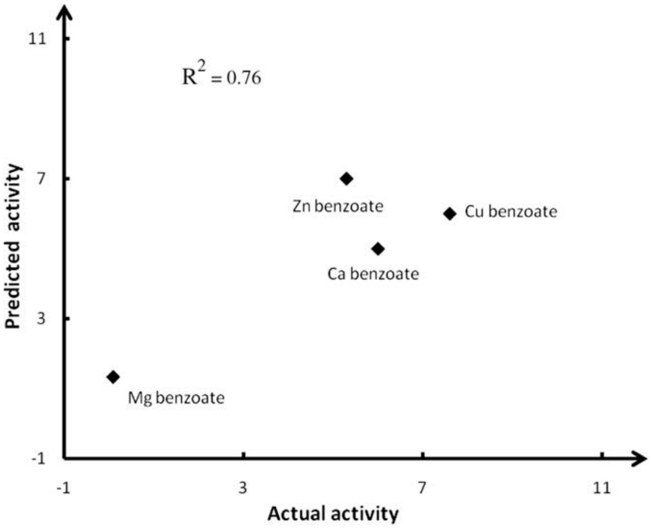

Fig. 1. Predicted with PLS-1 model versus actual antimicrobial activity showed by studied benzoates against *B. subtilis* after 24 h of incubation.

PLS reduces the number of predictors to a set of uncorrelated components and performs least squares regression on these components. Band wavenumbers (predictors) used to construct the PLS model were the same as for the models we have created before for another set of compounds: asymmetric and symmetric stretches of carboxylic anion, asymmetric deformations of carboxylic anion and 8a and 19b ring vibrations [7]. The response was antimicrobial activity. In Table 1, wavenumbers of 8a ring vibration are in the first row, while wavenumbers of 19b ring vibration are in the fourth row. This is of great importance that both previously and in this work we are statistically analyzing wavenumbers of bands assigned to the same vibrations. That confirms the proper selection of bands originating from the same bonds, involved in the model. Wavenumbers of those bands correlate with the antimicrobial activity of compounds. Therefore, the claim of determining antimicrobial activity by molecular structure (characterized by spectral parameters) is supported. However, as we have already stated, wavenumbers of carboxylate anion or ring vibrations taken into account separately did not produce satisfactory correlation. That means structure of whole molecule, but not anion or ring alone is of importance.

In the model proposed herein, validation with leave-one-out procedure was performed. Obtained statistical model is valid for current data, with correlation coefficient (R^2) equal to 0.81, 0.70 and 0.76, for *E. coli, B. subtilis* and *P. anomala*, respectively. Comparison of log(PRESS) values (see Table 5) allowed selection of strongest model out of one-, two- and three-factor models. High correlation coefficient confirm good correlation between spectral and biological data (current data), while moderately strong prediction strength is depicted by relatively high PRESS value. This weakness of proposed models is probably a result of a small number of data involved in the experiment, as well as error in relatively high confidence intervals of biological data.

4. Conclusions

The structure of carboxylic anion of solid state samples of the studied compounds is of different type: chelation structure in the case of Mg benzoate, bridging structure in the case of both Ca and Cu benzoates and monodentate structure in the case of Zn carboxylates which is proved by Δ value obtained for every compound. Electronic charge distribution within carboxylic anion is therefore different in all studied molecules, and slight differences occur in the aromatic ring.

In the case of *E. coli*, for solid state samples statistical model created with PLS technique produced good relation between spectral data and antimicrobial activity. Comparable results had been obtained in the past for another series of compounds and the same micro-organism [14]. However, even though the present model fits current data properly ($R^2 = 0.83$), its predictive strength is only moderately strong. Considering the lack of such relation for remaining salts, we suggest that the defense system of *E. coli* might be significantly different from other micro-organisms' defense systems, or the mode of action of the studied compounds against *E. coli* involve different properties of the molecule.

In case of spectra of water solutions model with statistically significant correlation coefficients and low log(PRESS) value was obtained for three out of four studied micro-organisms. Importance of both carboxylic anion and ring structure determining compound antimicrobial activity is confirmed by spectral region selection. Bands generated from vibrations of both carboxylic anion and ring are located in the used spectral regions. If other regions were analyzed alone or added to be analyzed together with selected carboxylic anion and ring vibrations, correlation coefficient dropped dramatically. Different number of factors involved in model calculated for different micro-organisms suggests somewhat different mode of action against studied micro-organisms. However, it is very likely that the most important factor, as regards antimicrobial activity and mode of action, is the ability of anion to coordinate hydrogen cations and therefore, at least partially, regulate pH value of the medium. In other words, buffer capacity of the solution of studied benzoates, which depends on both salt concentration and stability constants of weak acid incorporated in the salt, which is in turn determined by electronic charge distribution, is the factor regulating pH value of the medium. Therefore we suggest molecular structure, characterized by IR data, to be indirectly responsible for antimicrobial properties of given compound.

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