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Micellar electrokinetic chromatography with bile salts for separation of flavanone diastereomers

Abstract: *Sodium cholate and sodium deoxycholate were applied in the micellar electrokinetic chromatography technique as separating agents for the diastereomers of flavanone glycosides. The effects of the organic solvent buffer modifier, bile salt type and concentration were evaluated for enhancing distereomer separation. The critical micelle concentration of sodium cholate and sodium deoxycholate in the applied running buffer was determined. The binding constants of flavanone glycosides to bile salts micelle were estimated. The elaborated analytical method was used to analyse the flavanone fraction of bitter orange (Citrus aurantium) and grapefruit (Citrus paradisi) juice.*

Key words: *Bile salts, diastereomers separation, flavanone glycosides, MEKC, binding constants*

Streszczenie: *Cholan sodu i deoksycholan sodu zostały zastosowane w technice micelarnej elektrokinetycznej chromatografii cieczowej jako czynnik rozdzielający diastereomery glikozydów flawanonów. Oceniono wpływ rozpuszczalnika organicznego jako modyfikatora buforu, typu soli żółciowej oraz jej stężenia na wzmocnienie rozdzielenia diastereomerów. Wyznaczono krytyczne stężenie micelame cholanu sodu i deoksycholanu sodu w zastosowanym buforze rozdzielającym. Oszacowano stałe trwałości glikozydów flawanonów z micelami soli żółciowych. Opracowana metoda została wykorzystana do analizy frakcji flawanonowej w soku gorzkiej pomarańczy (Citrus aurantium) i grejfruta (Citrus paradise).*

Słowa kluczowe: *Sole żółciowe, rozdzielanie diastereomerów, glikozydy flawanonów, MEKC, stałe trwałości*

1. Introduction

Flavonoids are one of the largest groups of naturally occurring phenols and are widespread in the plant kingdom. Due to their pharmacological role and their health benefits, the analysis of flavonoids is of great importance [1, 2]. Flavanones belonging to the flavonoid group occur in relatively large quantities in citrus fruits. Due to the asymmetric C2 position in their moiety, flavanones are chiral compounds and thus can exist in two enantiomeric forms. As their glycosides have additional optically active sugar residue, they appear as a pair of diastereomers. Most probably in nature they are synthesized in (-)-2S configuration but during the plant growth or isolation procedure they may undergo partial or complete diastereomerization and racemization [3].

Capillary electrophoresis (CE) has been proposed as a complementary technique to reversed-phase high performance liquid chromatography (RP HPLC) for the separation of flavanone glycoside diastereomers [4-6]. In comparison with HPLC, CE is characterized by higher resolving power. Although the separation of diastereomers of flavanone glycosides in an achiral environment is theoretically possible, until now they were separated only with the use of a chiral agent. The summary of various chromatographic and electrophoretic methods applied for separation of chiral flavonoids including diastereomers of flavanone glycosides is presented in the review [7].

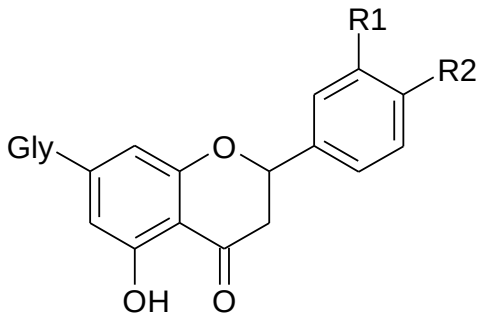
Among many surfactants able to form a micellar structure, bile salts play an important role, since they are biosurfactants in mammals and important cholesterol end products. Due to their rigid steroidal structure with hydrophobic and hydrophilic faces, their aggregation behaviour and micellar structure is different from that of traditional linear surfactants. One consequence of planar polarity is that bile salts in aqueous systems form smaller micelles than classical surfactants, with an aggregation number of 2-9 molecules [8]. Micelles of various surfactants are applied in a particular mode of capillary electrophoresis – micellar electrokinetic chromatography (MEKC). Since bile salts are chiral, their micelles have been applied in the separation of enantiomers in the MEKC technique [9, 10].

Our preliminary studies have shown that sodium cholate (NaC) as a chiral surfactant can be useful in the separation of selected flavanone glycosides [11]. In the current work, diastereomers of flavanone-7-O-glycosides were separated by micellar electrokinetic chromatography (MEKC) using sodium cholate (NaC) and sodium deoxycholate (NaDC) as additives in the background electrolyte. The effect of the buffer, organic modifier and concentration of the bile salt additive on the migration time and resolution was studied. The binding constants of flavanone glycosides to bile salts micelle were estimated. The elaborated analytical method was used to analyse the flavanone fraction of bitter orange (*Citrus aurantium*) and grapefruit (*Citrus paradisi*) juice.

2. Materials and methods

2.1. Investigated compounds

The structures of the investigated flavanone glycosides are presented in Fig. 1. They are composed from aglycone part and sugar residue. Neohesperidoside, rutinoside and glucoside comprise sugar moiety while isosakuranetin (in poncirin and didymin), hesperetin (in hesperidin and neohesperidin), naringenin (in naringin and narirutin) and eriodictyol (in eriocitrin, neoeriocitrin and pyracanthoside) are the aglycone parts.



Compound	R1	R2	Gly
Poncirin	H	OCH_3	O-Nh
Didymin			O-Ru
Neohesperidin	OH	OCH_3	O-Nh
Hesperidin			O-Ru
Narirutin	H	OH	O-Nh
Naringin			O-Ru
Neoeriocitrin	OH	OH	O-Nh
Eriocitrin			O-Ru
Pyracanthoside			O-Glu

Fig.1. The structures of the investigated flavanone glycosides; Nh –neohesperidoside, Ru – rutinoside, Glu – glucoside.

Naringin is the main bitter constituent of grapefruit (*Citrus paradisi*), narirutin and hesperidin are the constituents of sweet orange (*Citrus sinensis*), eriocitrin occurs in lemon (*Citrus limon*), neohesperidin, neoeriocitrin and poncirin are found in sour orange (*Citrus aurantium*), didymin was isolated from the leaves of bee balm (*Monarda didyma*), while pyracanthoside is a constituent of the bark of the apple tree (*Malus domestica*) [12]. It is generally accepted that flavanones are synthesized in nature in the S form. The occurrence of the R form in plants is a result of nonenzymatic racemization or epimerisation. Flavanone glycosides are weak acids with pKa values in the range of 9-10 [4].

2.2. Chemicals

Sodium cholate, sodium deoxycholate and sudan III were purchased from Fluka (Buchs, Switzerland) and were used as received. Naringin, narirutin, hesperidin, neohesperidin, didymin poncirin and pyracanthoside were obtained from Roth (Karlsruhe, Germany). Eriocitrin and neoeriocitrin were purchased from Extrasynthese (Genay, France). Methanol, 2-propanol, acetonitrile, $Na_2B_4O_7$, NaH_2PO_4 were purchased from Polskie Odczynniki Chemiczne (Gliwice, Poland).

All the other reagents used in preparing the buffer solution were of analytical grade and used without further purification.

2.3 Apparatus and Procedure

All the separations were performed on a Beckman CE System (P/ACE CE System, Fullerton, CA, USA) equipped with a diode array detector (DAD). The scan range was 190-300 nm and the electropherograms were stored at 280 nm. A fused-silica capillary with dimensions 75 μm i.d. x 58 cm (48

cm effective length) was thermostated at 25°C. The new capillary was washed with 1 M HCl for 5 min than with water for 2 min following by 0.1 M NaOH for 10 min and finally with water for 2 min. The applied voltage was 20kV. Before each set of measurements the capillary was flushed with 0.1 M NaOH for 2 min, then water for 2 min, and finally with the running buffer for 1.5 min.

Samples were injected by applying pressure of 0.75 psi for 3 s. All sample solutions and buffers were filtered through 0.45 µm polypropylene syringe filters prior to use. Methanol and Sudan III were injected to determine the electroosmotic flow (EOF) and the migration time of micelles, respectively.

2.4. Sample preparation

Sour orange and grapefruit juice samples were prepared similarly as in the procedure described previously [13]. 5 ml of orange or grapefruit juice was applied to a DPA-6S SPE (6 ml tube, 500 mg) cartridge of Supelco (Bellefonte, PA, USA). After washing with 10 ml of water and drying, the flavanones were extracted with 1 ml of methanol.

3. Results and discussion

3.1. Effect of organic solvent buffer modifier

The pH of the running buffer was chosen as 7.5, below the expected pKa of flavanones to increase possibility of interaction of non-ionised flavanones with anionic micelles of cholates. Three running buffers were tested: tertraborate/borate (25 mM $Na_2B_4O_7$/200 mM H_3BO_3), phosphate (50 mM Na_2HPO_4/50 mM NaH_2PO_4) and borate/phosphate (50 mM $Na_2B_4O_7$/200 mM NaH_2PO_4). Finally, the borate/phosphate buffer was chosen for further optimisation as giving overall the best resolution properties in the case of NaC and NaDC.

Organic solvents are often used as modifiers of the running buffer to increase the resolution of closely migrating analytes. They usually reduce electroosmotic flow (EOF). At high concentration, the organic solvent may lead to micelle breakdown.

Our studies investigated the addition of three organic solvents: methanol, 2-propanol and acetonitrile at concentration of 10%. At this concentration their influence on the CMC is rather small and can be neglected [14].

Table 1 presents the electrophoretical parameters obtained for three selected flavanones in buffers with the investigated organic additives for NaC and NaDC. For comparison, electrophoretic measurements were performed with a buffer without organic modifier for the NaC micellar system. Since the solubility of NaDC in a running buffer without organic modifier was insufficient to prepare a stable solution, it was impossible to perform similar comparative measurements for this micellar system.

Table 1 Influence of organic solvent on diastereoseparation process of selected flavanones; conditions: pH 7.5, temp.25^{0}C, capillary: 58 cm × 75 µm ID, voltage 20kV, detection 280 nm.

Flavanone		**40 mM NaC/50 mM borate/200 mM phosphate**				**100 mM NaDC/50 mM borate/200 mM phosphate**		
		0%	10% ACN	10% 2-PrOH	10%-MeOH	10% ACN	10% 2-PrOH	10%-MeOH
Naringin	t_{m2}[1]	10.84	10.19	14.65	16.20	18.61	21.40	28.49
	α_{app}[2]	1.024	1.011	1.013	1.025	1.007	1.003	1.046
	R_s[3]	1.60	0.86	1.50	2.19	0.68	0.10	1.16
Neohesperidin	t_{m2}	10.80	10.19	14.59	16.48	18.66	21.71	28.75
	α_{app}	1.025	1.012	1.016	1.030	1.009	1.006	1.014
	R_s	0.90	0.72	1.50	2.72	0.89	0.81	1.62
Neoeriocitrin	t_{m2}	8.89	10.35	14.29	14.64	13.37	18.77	17.93
	α_{app}	1.008	1	1.004	1.007	1.006	1.007	1.010
	R_s	0.83	0	0.28	0.56	0.58	0.86	0.90
EOF	t_m (μ_{eof})	6.00 (2.32)	7.20 (1.93)	9.50 (1.45)	8.90 (1.56)	8.0 (1.74)	10.0 (1.39)	9.40 (1.48)

[1] t_{m2}- migration time of second migrated diasteromer [min],

[2] $\alpha_{app} = t_1 / t_2$ – separation factor, R_s- resolution;

[3] μ_{eof}- electrophoretic mobility of buffer [10^{-4}cm^{2}V^{-1}s^{-1}]

The organic additive can influence the double electric layer on the capillary wall and the physicochemical properties of the running buffer (e.g. viscosity) as well as the stability and selective properties of the micelle.

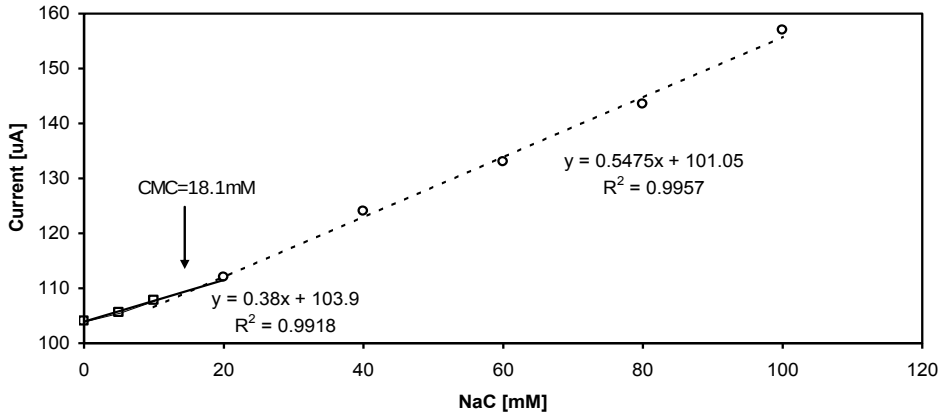
All the investigated organic solvents reduce the EOF of the solution buffer/NaC. Among them, 2-propanol reduces the EOF the most effectively, while acetonitrile has a very small effect on electroosmotic velocity ($\mu_{eof}^{0} > \mu_{eof}^{ACN} > \mu_{eof}^{MeOH} > \mu_{eof}^{2-\mathrm{Pr}OH}$). The longest migration times are observed for the buffer with added methanol. This solvent also gives the highest resolution and separation factor for the flavanones under consideration. Interestingly, the migration times of naringin and neohesperidin for NaC/acetonitrile are slightly shorter than for the buffer without the addition of an organic solvent. In this case the diastereomer resolution is the worst, and for neoeriocitrin is not even observable. It seems that acetonitrile unfavourably modifies the micelle, making it less convenient for interaction with flavanones. As methanol gives the best resolution of the selected diastereomers, this organic solvent was chosen for further optimisation of the method.

3.2. Determination of critical micelle concentration

Critical micelle concentration (CMC) is an important value indicating the range of concentrations in a solution where the surfactant starts to form micellar structures. Since the CMC is dependent on the solvent and other dissolved species, it is important to determine this value under given experimental conditions (in the running buffer solution). Among various methods used to determine the CMC, the current-based method was applied due to its suitability and simplicity. In this method the electric current as a function of surfactant concentration at a given electric field is measured using CE apparatus [15]. An increase in conductivity is observed when the surfactant concentration exceeds the CMC. Thus the plot of electric current versus surfactant concentration consists of two lines of different slopes corresponding to the monomer and micellar state. The intersection of these lines defines the CMC.

The plots of the relation of observed current intensity vs. bile salt concentration are presented in Fig. 2. The CMC values determined from the plots are 18.1 mM for NaC and 7.1 mM for NaDC. These values are in good agreement with those determined by other methods [16].

a)



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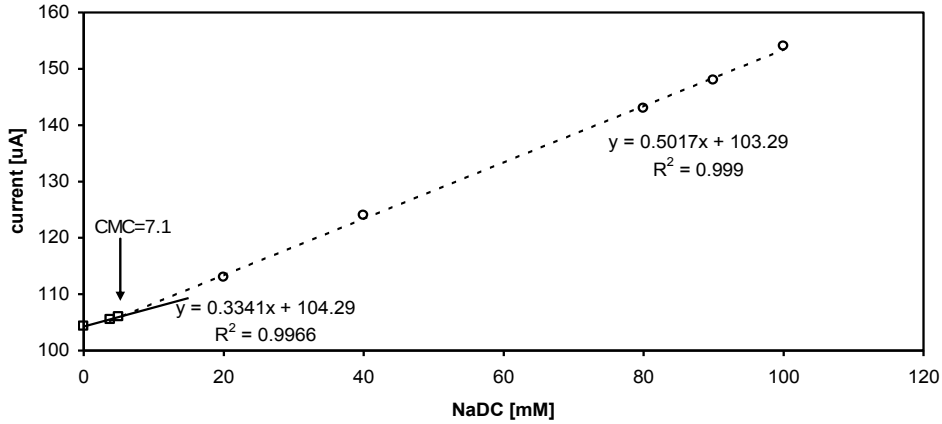


Fig. 2. The relation of current intensity vs. NaC (a) and NaDC (b) concentrations obtained under MEKC conditions. Running buffer: 50 mM borate/200 mM phosphate buffer, pH 7.5, 10% of methanol; voltage: 20kV.

3.3. Effect of bile salt concentration

The most important factor influencing the resolution in the studied system is the concentration of bile salts. Fig. 3 presents electropherograms of neohesperidin obtained at various NaC concentrations.

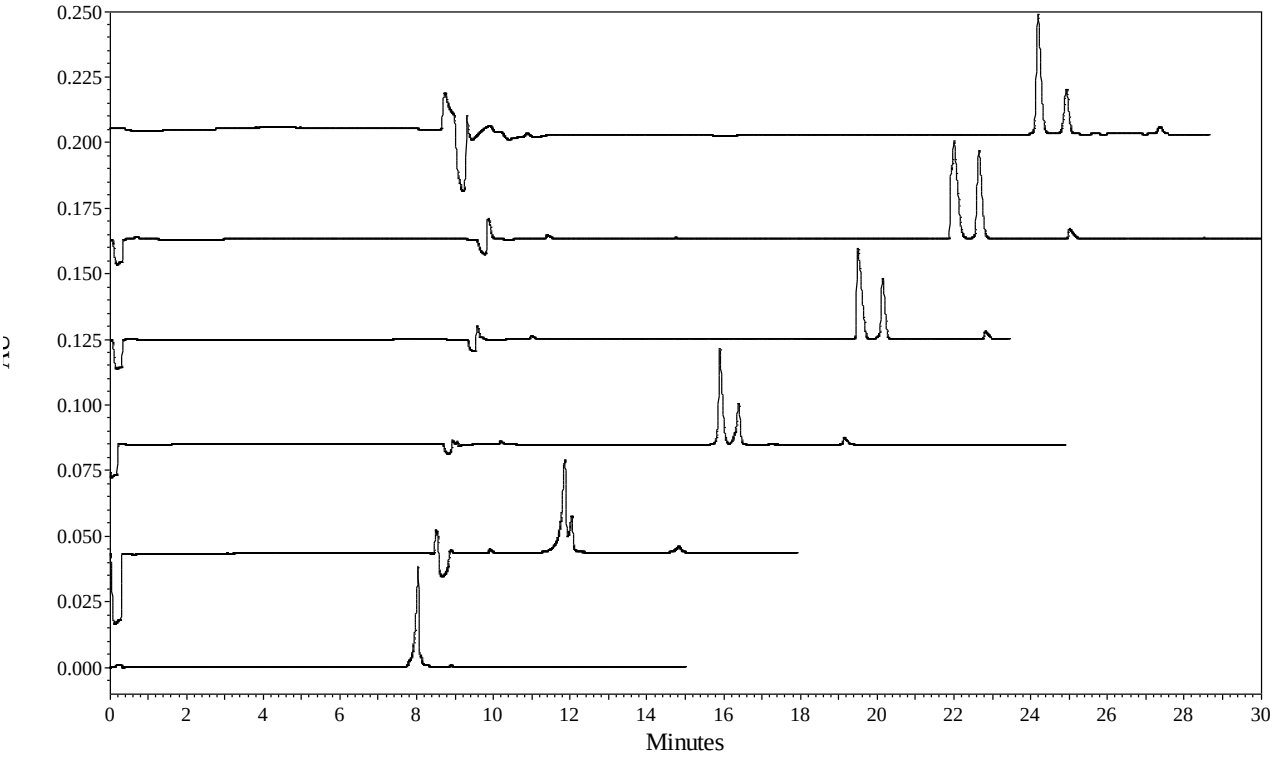
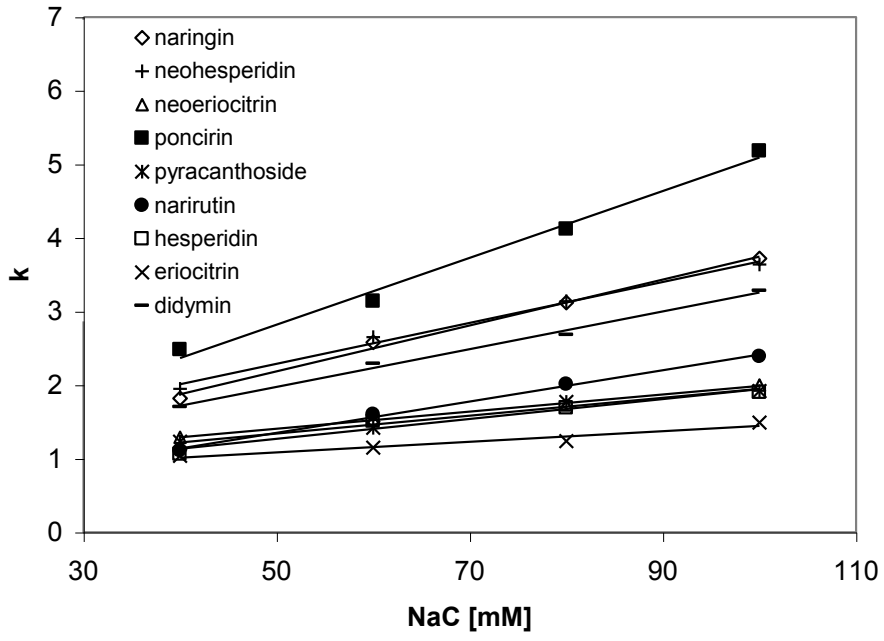


Fig 3. Electropherograms of neohesperidin diastereomers separation obtained at various NaC concentrations. Running buffer: 50mM borate/200mM phosphate buffer, pH 7.5, 10% of methanol; voltage: 20kV.

The increase of bile salt concentration results in the increase of migration times and t_{eof}. Since the investigated flavanones are non-ionised under the studied conditions, the retention factor (instead of mobility) was presented as a function of concentration. The influence of NaC and NaDC concentration on the retention factor of the slower-migrating diastereomers of flavanones is presented in Fig. 4.

a)



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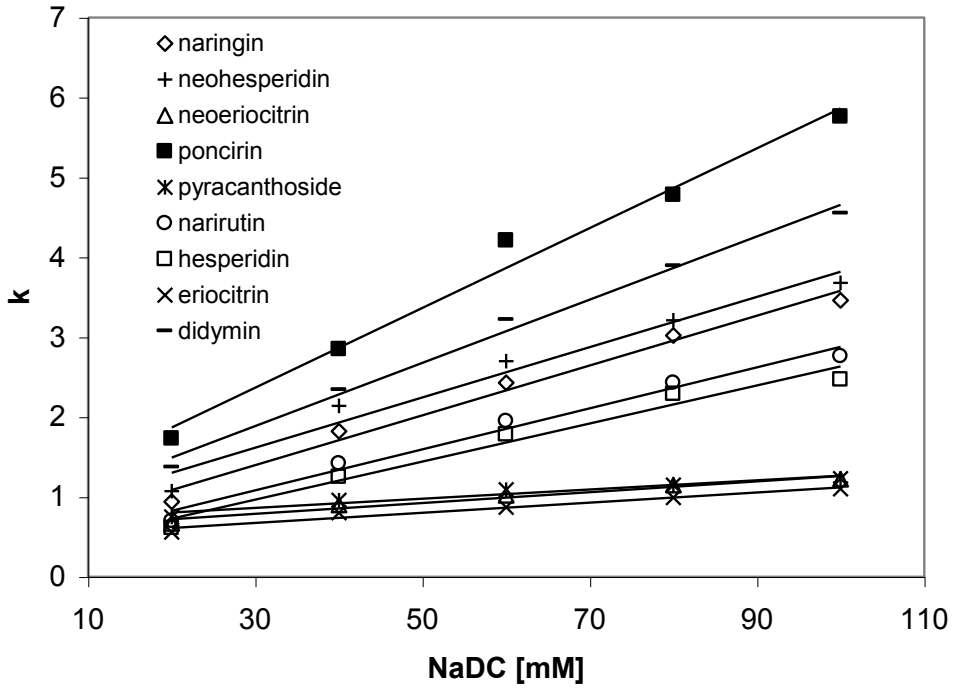


Fig. 4. The dependence of retention factor from NaC (a) and NaDC (b) concentrations obtained for the studied flavanones.

The retention factors were calculated from the equation:

$$k = \frac{t_e - t_{eof}}{t_{eof}\left(1 - \frac{t_e}{t_{mc}}\right)} \quad [1]$$

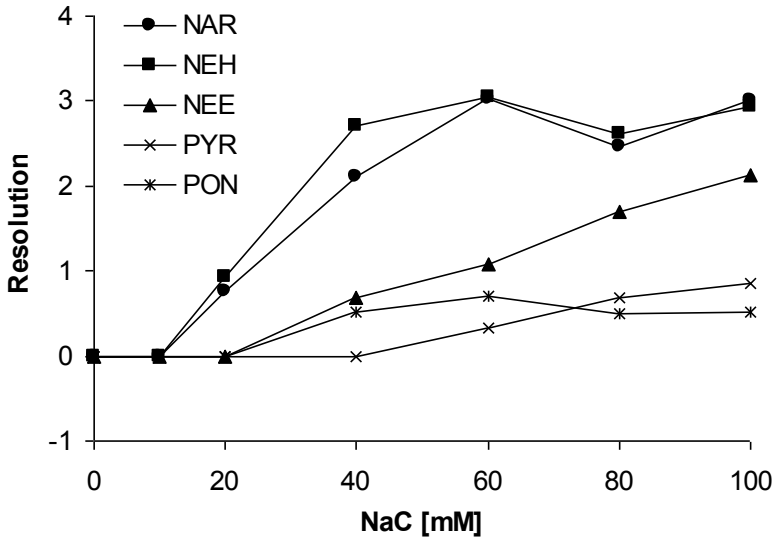
where t_e, t_{eof} and t_{mc} are the migration times of the analyte, the unretained solute moving at the EOF rate and the micelle, respectively.

The retention factor was not calculated for concentration of 10 and 20 mM of NaC and 10 of NaDC, since it was not possible to determine the mobility of the surfactant. The method applied in the present paper to measure the mobility of Sudan III as a micelle marker was not efficient below the CMC and in its vicinity.

The retention factors are practically linearly dependent on NaC and NaDC concentration. The slope is proportional to the interaction between micelle and flavanone.

Fig. 5 illustrates the effect of NaC and NaDC on the resolution Rs of the flavanone diastereomers. For flavanones with eriodictyol as the aglycone (neoeriocitrin and pyracanthoside), Rs grows almost linearly in the whole studied concentration range, while for the rest of the compounds Rs grows relatively fast and reach a plateau at about 60 mM.

a)



b)

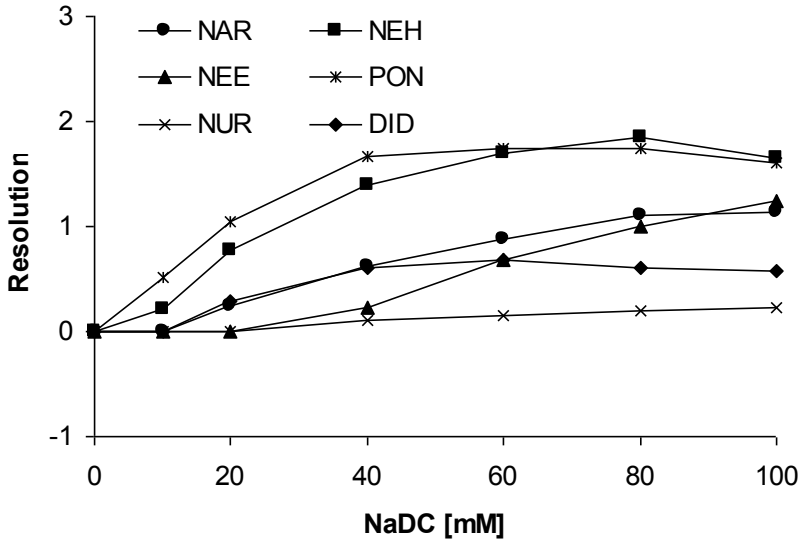


Fig. 5. The relation of resolution vs NaC (a) and NaDC (b) concentration obtained for separated diastereomers of flavanones.

The micelle of NaC enables the separation of naringin, neoeriocitrin, neohesperidin, poncirin - flavanone glycosides with neohesperidose moiety and pyracanthoside with glucosidic moiety. The separation of diastereomers with rutinosidic moiety is not observed for NaC. The micelle of NaDC recognizes diastereomers of flavanones with neohesperidosidic moiety and also two flavanones with rutinoside – didymin and narirutin.

The micelle of NaC is more effective in separating neohesperidosidic diastereomers, however the micelle of NaDC also recognizes two diastereomers from the rutinoside group.

3.4. Estimation of binding constants

MEKC with various bile salts as micellar agents were applied for estimation of binding constants between bile salts and drugs [17], proteins [18] and enantiomers of 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate [19]. The presented models describe interactions between negatively charged micelles and charged analytes. Since investigated flavanones are non-ionised under applied pH the simplified model of interactions is proposed basing on the model described by Saitoh and al. [18].

The retention factor k can be related to the distribution coefficient K of a solute S between micellar and aqueous phase through:

$$k = K\frac{V_{mic}}{V_{aq}} \quad [2]$$

where V_{mic} and V_{aq} are volumes of micellar and aqueous phases, while distribution coefficient K is given by the relation of concentrations of a solute in micellar (S_{mic}) and aqueous phase (S_{aq}):

$$K = \frac{[S]_{mic}}{[S]_{aq}} \quad [3]$$

The concentration of micellar phase is equal to difference between bile salt concentration B and CMC. Thus the relation can be written as follows:

$$k = K\frac{\bar{v}(B-CMC)}{1-\bar{v}(B-CMC)} \quad [4]$$

where $\bar{v}$ is partial specific volume of micelle.

Since the volume fraction of micellar phase is very small ($1-\bar{v}(B-CMC)\approx 1$) thus the equation 3 can be simplified to:

$$k = K\bar{v}(B-CMC) \quad [5]$$

The binding constant K_B of solute-micelle interaction can be defined as follows:

$$K_B = \frac{[S_{bound}]}{[S_{free}][B-CMC]} \quad [6]$$

where S_{bound} and S_{free} are concentrations of a solute bounded with bile salt and free solute.
S_{aq} can be represented by S_{free}:

$$[S]_{aq} = \frac{[S_{free}]}{V_{aq}} = \frac{[S_{free}]}{1-\bar{v}[B-CMC]} \approx [S_{free}] \quad [7]$$

Similarly S_{mic} can be represented by S_{bound}:

$$[S]_{mic} = \frac{[S_{bound}]}{V_{mic}} = \frac{[S_{bound}]}{\bar{v}[B-CMC]} \quad [8]$$

By substitution of equations 7 and 8 to equation 3, we obtain:

$$K = \frac{[S_{bound}]}{\bar{v}[S_{free}][B-CMC]} = \frac{K_B}{\bar{v}} \quad [8]$$

Finally the following relationship is obtained:

$$k = K_B[B-CMC] \quad [9]$$

The binding constants of flavanones to NaC and NaDC were estimated from the slopes of linear relationship of k vs. [B-CMC]. The obtained data are presented in table 2.

Table 2. Binding constants of flavanones to bile salts

Compound		NaC	NaDC
Naringin	S	29.1±1.3	30.6±2.3
	R	31.1±1.6	31.2±2.3
Neohesperidin	S	26.3±1.4	30.7±3.2
	R	27.8±1.8	31.4±3.4
Neoeriocitrin	S	10.7±0.5	6.4±0.7
	R	11.7±0.5	6.7±0.8
Poncirin	S	44.7±3.2	49.9±3.6
	R	45.4±3.2	49.0±3.4
Narirutin	S	21.4±0.9	25.3±2.0
	R		25.6±2.1
Hesperidin	S	13.5±2.2	23.8±2.4
	R		
Eriocitrin	S	7.0±1.2	6.3±0.7
	R		
Didymin	S	25.6±1.5	39.5±2.1
	R		39.1±2.0
Pyracanthoside	S	12.2±1.0	5.8±0.8
	R	13.0±1.0	

The estimated binding constants have values between 6 and 50 M. Independently on the kind of bile salt the binding constants are higher for neohesperidosides than for rutinosides. More hydrophilic flavanones (eriocitrin, neoeriocitrin, pyracanthoside) stronger interact with less hydrophobic NaC than NaDC. More hydrophobic flavanones (poncirin, didymin, hesperidin, neohesperidin) form stronger complexes with less hydrophilic NaDC than with NaC.

3.5. Practical applications

The elaborated methods were applied in practice to analyse the flavanone fraction of bitter orange (Citrus aurantium) and grapefruit (Citrus paradisi) juice. The main components of the flavanone fraction of bitter orange are neoeriocitrin, naringin and neohesperidin, while naringin with small amounts of narirutin and neohesperidin are the main flavanones of grapefruit. Fig. 6a presents the electropherograms of the flavanone fraction of sour orange juice obtained with buffers containing 100 mM of NaC and NaDC. Comparing the selective properties of NaC and NaDC, it is observed that although NaC is more selective towards diastereomers it does not separate naringin and neohesperidin, while NaDC allows the separation of diastereomers as well as all the flavanones found in bitter orange.

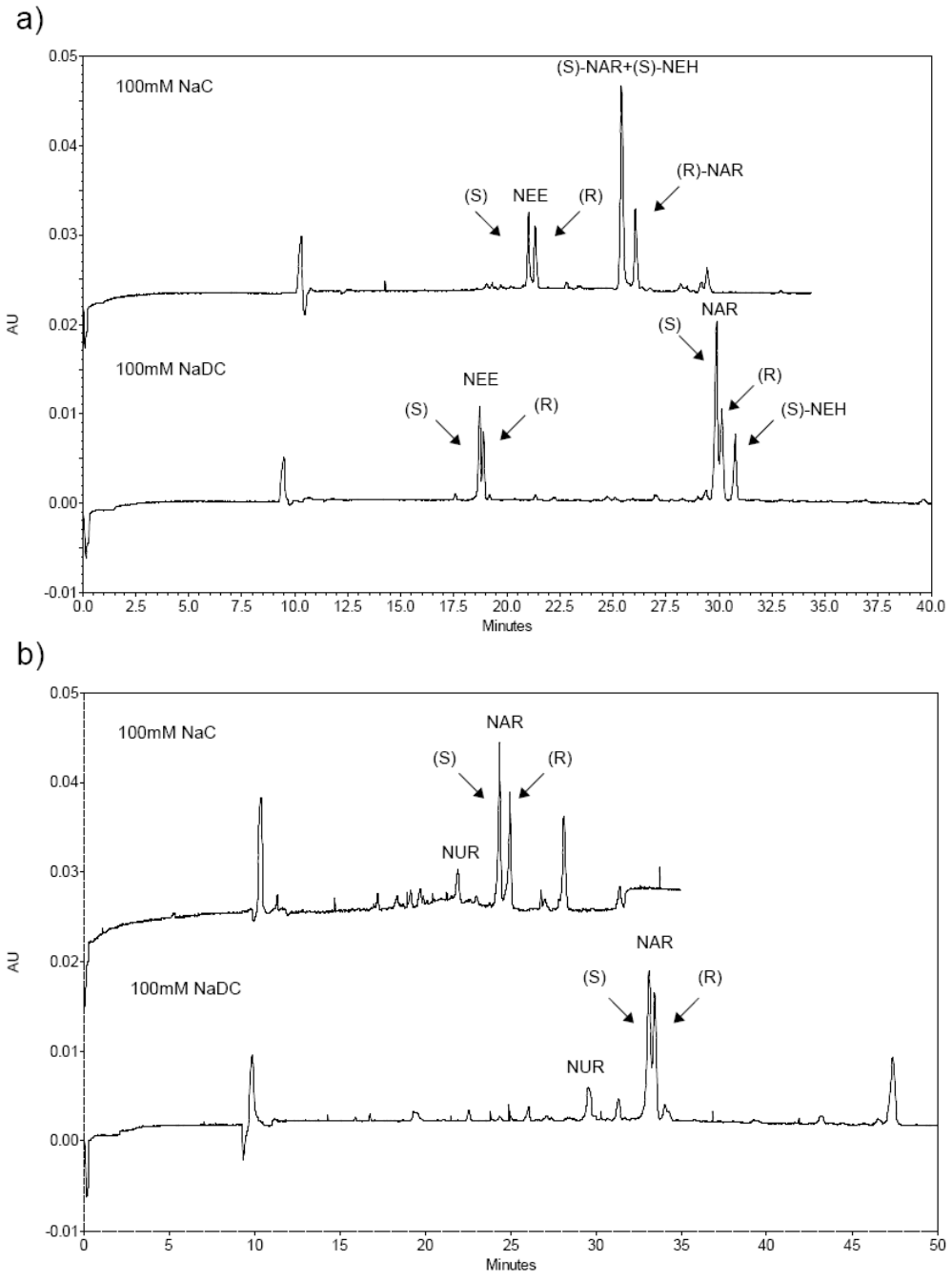


Fig. 6. Electropherograms of the flavanone fraction of sour orange juice (a) and grapefruit juice (b) obtained with buffer 50 mM borate/200 mM phosphate buffer, pH 7.5, 10% of methanol containing 100 mM of NaC and NaDC.

The diastereomeric ratio 2S:2R of neoeriocitrin and naringin is 59:41 and 66:34, respectively, while neohesperidin appears only in 2S form. The applied buffer seems more suitable than that used by Gel-Moreto et al. [6] since very mild pH (7.5) does not promote the diastereomerization reaction [20]. In fact, the obtained diastereomeric ratio of flavanones (2S:2R) is higher than those obtained by Gel-Moreto et al.

Electropherograms of the flavonoid fraction of grapefruit juice under the same conditions as in the case of bitter orange juice are presented in Fig. 6b. The main component of grapefruit juice is naringin with small amounts of narirutin and neohesperidin. The ratio of the S:R form of naringin was 55:45.

4. Concluding remarks

The micelles of NaC and NaDC are effective resolving agents for diastereomers of flavanone glycosides with neohesperidosidic moiety. Although NaC exhibits better selective properties towards diastereomers of flavanones, NaDC appears to be more effective for separating a mixture of flavanones from citrus juices. The moderate diastereoselective properties of NaDC are accompanied by better selective properties towards the group of flavanones than those of NaC.

5. References

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