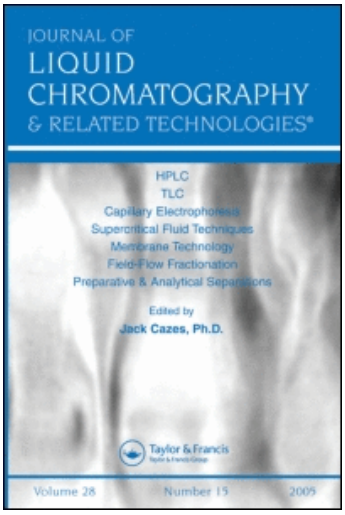

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Abstract: The pH role in benzoic acid derivatives chromatographic analysis were under the current study. The retention factor (k), as well as the peak asymmetry (f_{AS}), were determined and compared using five commercially available reversed phase columns. Utilization of statistical approaches of tested chromatographic columns were divided into several groups according to the obtained data (retention factor, asymmetry) due to the different mobile phases pH (pH = 5.8 and 3.0). Although four studied columns (Purospher-STAR, Alltima, Reprosil, Symmetry) were the octadecyl type, they showed significant differences in the benzoic acids derivatives. Obtained results allowed for distinctions in individual column chromatographic behavior explanation.

Keywords: High performance liquid chromatography, Octadecyl stationary phases, Benzoic acid derivatives, Retention mechanism, Columns partition, Cluster analysis

INTRODUCTION

The stationary phases must be characterized for quality control purposes. Often obtained results indicate differences in columns which are supposed to be

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identical, packed with the same stationary phase, e.g., octadecyl types. Commercially available columns, even produced by the same manufacturer exhibit different chromatographic properties.[1,2] Determining the structure and chromatographic properties of used stationary phases is important because of the description of the retention mechanism. Chromatographic separation is the consequence of differences in the analyzed compound affinities to the stationary and mobile phase. One of the ways of improving the separation results is the change around the mobile phase, e.g., pH of the mobile phase.[3] Such change may influence stationary phase and/or analyte character. Among substances, of which the character is changing with the change of solution pH are compounds investigated in the present study—benzoic acid derivatives. They belong to phenolic acids. Recent interest in these substances stem from their potential protective role against oxidative damage diseases (coronary heart disease, stroke, and cancers) through ingestion of fruits and vegetables.[4,5] Although these compounds differed only in the number and position of the hydroxy or carboxylic groups, they exhibited quite different properties.[4] Their biological importance (antioxidant character) requires development of good determination methods for these substances in natural samples. The most popular final determination method is high performance liquid chromatography (HPLC).[6–9] Although chromatographic columns used for the analysis of phenolic acids are often of similar types, the obtained results very often differ between one another. The main reason for such effects are dissimilarities among the column packing materials. The silica type used for the stationary phase preparation affect the chromatographic retention and, as a consequence, the reproducibility of chromatographic data.[10] This problem concerns even highly purified silica supports, including those considered to be the least acidic ones.[11] The information provided by the column manufacturers are frequently insufficient in the case of choosing the proper packing material for analysis of our substances. There are many different physico-chemical methods for column characterizing.[1] However, those techniques require damaging of the column before chromatographic analysis. In the case of commercial columns, common methods for their evaluation are different types of chromatographic tests.[1]

Ionic compounds may interact with the stationary phase in a specific way. Chemically modified silica gel stationary phases contain residual silanols, which are ionized in aqueous salt solutions, due to the pH value.[12] In the case of ionizable compounds (such as studied phenolic acids), chromatographic retention (k) is an average of the retention factors of ionized and non-ionized analyte forms as has been discussed elsewhere.[13,14] For acids:

$$k = f_{[HA]}k_{[HA]} + f_{[A^-]}k_{[A^-]} \tag{1}$$

$$f_{[HA]} = \frac{1}{K_a/[H^+] + 1} \tag{2}$$

$$f_{[A^-]} = 1 - f_{[HA]} \tag{3}$$

where: *f*-mole fractions of specific forms, K_a-dissociation constant, $[H^+]$-hydrogen ion concentration.

In the present study, the differences in four octadecyl and one embedded chromatographic column with an octadecyl hydrophobic segment have been investigated through the analysis of six benzoic acids derivatives (p-hydroxybenzoic acid, gallic acid, protocatechuic acid, vanillic acid, gentisic acid, syringic acid). As a consequence, dissimilarities were observed during the separation of analyzed compounds on used packing materials and in different pH of mobile phases. The column partitions have been performed on the basis of statistical approaches.

EXPERIMENTAL

Materials and Reagents

Standards of gallic acid (GA), p-hydroxybenzoic acid (pHBA) were purchased from Merck (Darmstadt, Germany), protocatechuic acid (PA) was obtained from the Research Institute of Food Industry (Biocentrum Modra, Bratislava, Slovakia), gentisic acid (GeA), vanillic acid (VA) from MGP (Brno, Czech Republic), and syringic acid (SyrA) was supplied by Fluka (Buchs, Switzerland). The structures and basic properties of analyzed acids are shown in Table 1. For the preparation of binary aqueous – organic mobile phases methanol of HPLC grade was obtained from Scherlau Chemie S. A. (Barcelona, Spain), as well as: deionized water from Milli-Q system (Millipore, El Passo, TX, USA), formic acid (Lachema, Brno, Czech Republic).

Standard Solutions

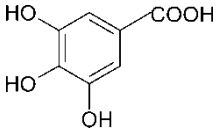
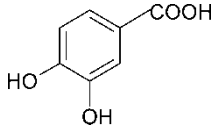
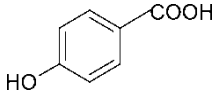
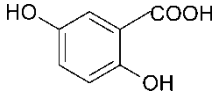
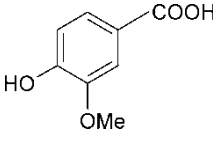
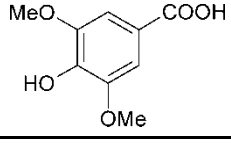
The stock solutions of all compounds from Table 1 (concentration 10 $\mu g/mL$) were prepared by dissolving pure standards in methanol and stored in a freezer at $-20^{\circ}C$. Working solutions were prepared by diluting the stock solutions with the mobile phase (ca.).

Apparatus and Chromatographic Conditions

Five commercial reversed-phase (C_{18}) columns were used: Alltima Rocket RP-18e (Alltech, Belgium), Purosphere® Star RP-18e (Merck, Germany), Reprosil 100-C18 (Maisch, Germany), Symmetry® C18 (Waters, USA), Synergi-4μ Fusion-RP-80A (Phenomenex, Canada). The physico-chemical properties of tested stationary phases are presented in Table 2.

An HP 1050 high performance liquid chromatography system (Hewlett-Packard, Waldron, Germany), with a quaternary pump, a Rheodyne 7125 manual injection valve (Rheodyne, Berkeley, CA, USA) with a 20 μL loop

Table 1. Structures, abbreviations, and properties of studied compounds

Phenolic acid	Abbreviation	Structure	pK_a[a]
Gallic acid	GA		4.41
Protocatechic acid	PA		4.48
p-Hydroxybenzoic acid	pHBA		4.48
Gentisic acid	GeA		2.97
Vanillic acid	VA		4.42
Syringic acid	SyrA		4.34

[a]The pKa values were taken from Refs. [15] and [16].

were selected for chromatographic measurements. HP ChemStation was used for data collection.

Elution was carried out with isocratic conditions of 70/30 v/v: (1) methanol and water (pure water with pH = 5.8) and (2) methanol/water (pH of water adjusted to 3.0 with addition of formic acid). The flow rate was 0.6 mL/min. Chromatograms were acquired at wavelengths of 254 and 340 nm.

For the interpretation of the results Statistica v. 5.1 packages for Windows (StatSoft, Tulsa, USA) were used.

RESULTS AND DISCUSSION

The presence of benzoic acid derivatives in plants and their biological importance make their determination evaluation neccessary. As high performance

Table 2. Chromatographic columns used in the study properties

Column	Dimensions (L × i.d., mm)	Particle size (μm)	Pore diameter (Å)	Pore volume (mL/g)	Specific surface area (m^2/g)	C-content (%)	End-capped
Alltima ROCKET RP-18e	54 × 7.0	3	100	—[a]	350	16.0	YES
Purosphere® STAR RP-18e	250 × 4.6	5	120	1.00	350	18.0	YES
Reprosil 100-C18	250 × 4.0	5	100	—[a]	300	15.0	YES
Symmetry® C18	150 × 3.9	5	100	—[a]	335	19.0	YES
Synergi-4u FUSION-RP-80A	150 × 4.6	4	80	1.05	475	—[a]	Polar embedded

[a]Data not available.

liquid chromatography is a simple, quick, and selective method, it is the most popular method for analyzing p-hydroxybenzoic acid, gallic acid, protocatechuic acid, vanillic acid, gentisic acid, and syringic acid determination. Almost all of the utilized procedures use commercial octadecyl columns and the mobile phase is acidic (using the acetic or formic acid).[6–9,17,18] The addition of acid into the mobile phase for analysis of phenolic acids by reversed phase high performance liquid chromatography (RP-HPLC) is carried out in order to improve the peak shapes of analytes, the resolution, and reproducibility of analyses. Moreover, the addition of acid leads to suppression of the ionization of phenolic and/or carboxylic groups. In this work, the separation of analyzed compounds on different octadecyl columns was performed for comparison of results and selection of the best one for the studies. It appeared that depending on the chromatographic column manufacturer there are different effects of separation, although all used packings were the same type. Figure 1 shows the example results of six analyzed compounds separation on two columns (Purosphere STAR, Symmetry C18)

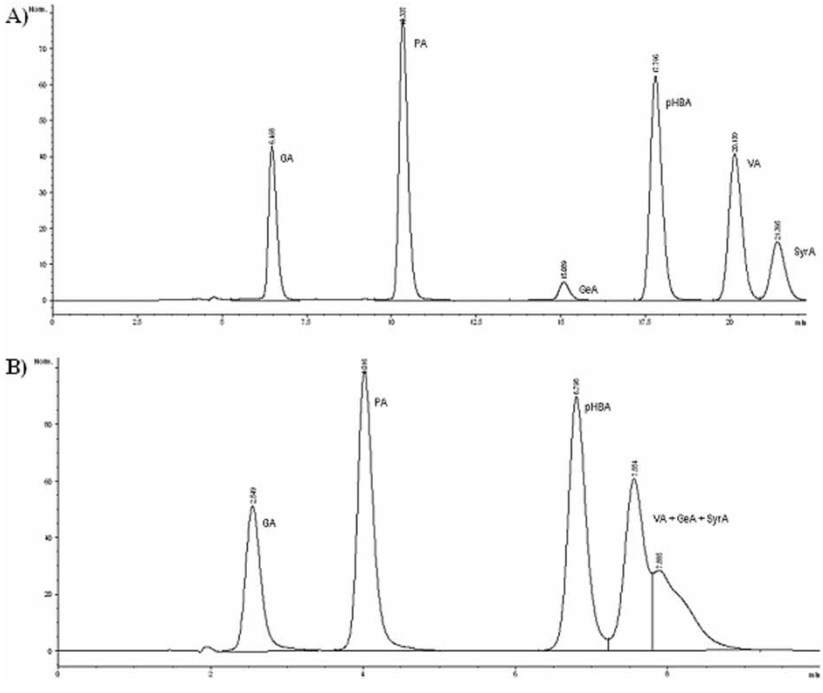


Figure 1. Separation of a mixture of benzoic acid derivatives at pH 3.0: A) Purospher STAR, B) on Symmetry C18. Mobile phase: 70/30 v/v methanol/water (pH of water was 3.0; adjusted with formic acid); flow rate of 0.6 mL/min; detection 254 nm. Notation: GA-gallic acid, PA-protocatechuic acid, pHBA-p-hydroxybenzoic acid, VA-vanillic acid, GeA-gentisic acid, SyrA-syringic acid.

under the same mobile phase conditions. Dissimilarity among the resolution and analysis time is obvious. Despite Symmetry C18 providing a shorter time of separation, the resolution was poorer. In the case of improving the resolution results, the mobile phase pH has been changed to 5.8, where addition of formic acid was skipped and the mobile phase consisted only of pure water and methanol. However, once again obtained results depended on the column used (Figure 2) and the resolution of analyzed acidic derivatives was different on two octadecyl columns. It seems that although alkyl-bonded silica columns are considered a well defined, highly precise, and the most successful analytical tool, the differences between these materials are still meaningful. RP-HPLC packing materials are made with porous silica prepared from different silicon products, using different processes, depending on the manufacturer. In addition they are bonded with different reagents, following different procedures. The stationary phases are similar and the retention mechanisms involved are the same. On the other hand, the detailed balance of the

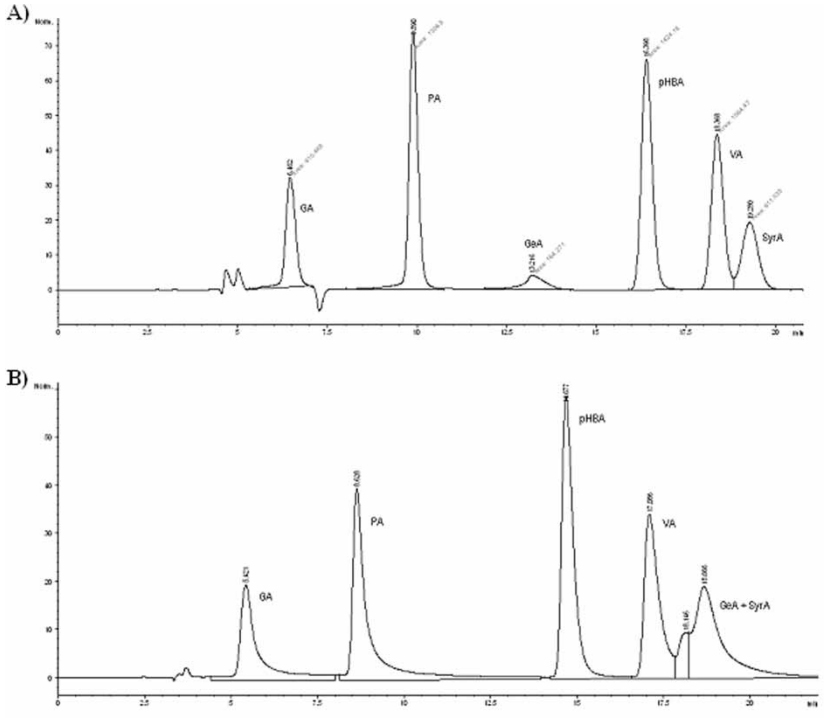


Figure 2. Separation of a mixture of benzoic acid derivatives at pH 5.8: A) Purospher STAR, B) Reprosil. Mobile phase: 70/30 v/v methanol/water; flow rate of 0.6 mL/min; detection 254 nm. Notation: GA-gallic acid, PA-protocatechuic acid, pHBA-p-hydroxybenzoic acid, VA-vanillic acid, GeA-gentisic acid, SyrA-syringic acid.

contributions of these mechanisms may differ in important ways on different brands of octadecyl columns. This explains the variety of the separation results for a given mixture. As it was concluded from presented results, replacing an certain brand of RP-HPLC column by various (even the same type) columns leads to significantly different separations.

Benzoic acids derivatives resolution results served as an initial observation of not only the role of chromatographic columns, but also mobile phase pH. In the case of pH investigation in benzoic acids retention, and as a consequence their potential as a chromatographic column characterizing, the retention coefficient and asymmetry were determined for all of the analyzed compounds on five columns in pH equal to 3.0 and 5.8. Obtained data are summarized in Table 3.

Obtained data presented in Table 3 (k values) were used for column comparison attempt purposes. The cluster analysis (CA) method was chosen for tests, which were carried out first with data standardization; next Ward's agglomeration method was chosen and the distance measurement was Euclidean. Received tree's graph enabled assembling of the columns into several clusters as it is presented in Figure 3. Figure 3A presents columns partition in $pH = 3.0$. The biggest similarities concern two columns: Symmetry C18 and Purosphere STAR RP-18e. Such assignment is probably the consequence of almost identical carbon content on the modified silica surface (Table 2). Through a similar hydrophobic character of those columns, the retention mechanism is also similar. The second cluster creates the remaining three packing materials, among which two, Alltima ROCKET RP-18e and Reprosil 100-C18, are the most similar. According to the manufacturer on the first stationary phase surface there is 15% of chemically bonded carbon, while on the second one 16%. Therefore, benzoic acids derivatives retention on those two packings is likewise similar. Assigning Synergi FUSION column to this cluster may seem to be surprising, because of its different surface structure. This column consist of octadecyl chains, however, there are also inbuilt polar groups, which decrease its hydrophobic character and, as a consequence, allow attaching polar embedded packing to the cluster with columns of lower hydrophobicity. Obtained results indicate that division in $pH = 3$ was done mainly on the basis of lipophilic character of the columns. The percentage of chemically bonded carbon allows for determination of part of residual silanols. Different mobile phase pH causes the alteration in surface silanols dissociation. Such effect is the reason for differences in column partition with the change in mobile phase pH.

Figure 3B presents results obtained in more basic pH (5.8). It is obvious that Synergi-FUSION-RP differs the most from the other columns, because of different stationary phase character. Greater retention of analyzed compounds in more basic pH is characteristic for this column in comparison with other columns. The reason for this situation is the presence of a mentioned polar

Table 3. Retention factor *k* and asymmetry f_{AS} for phenolic acids at pH 5.8 and pH 3.0 of water in the mobile phase (for conditions see text)

	Alltima ROCKET RP-18e				Purosphere® STAR RP-18e				Reprosil 100-C18				Symmetry® C18				Synergi-4u FUSION-RP-80A			
	pH = 3		pH = 5.8		pH = 3		pH = 5.8		pH = 3		pH = 5.8		pH = 3		pH = 5.8		pH = 3		pH = 5.8	
Solute	k	f_{AS}	k	f_{AS}	k	f_{AS}	k	f_{AS}	k	f_{AS}	k	f_{AS}	k	f_{AS}	k	f_{AS}	k	f_{AS}	k	f_{AS}
GA	0.45	1.4	0.39	1.3	0.46	1.3	0.42	1.2	0.53	1.5	0.62	2.8	0.39	1.5	0.45	2.5	0.63	0.6	1.04	1.6
PA	1.47	1.4	1.21	1.2	1.25	1.3	1.16	1.2	1.61	1.5	1.52	2.9	1.20	1.5	1.10	0.7	1.66	0.6	2.03	1.1
pHBA	3.44	1.4	2.45	1.3	2.76	1.2	2.60	1.1	3.68	1.2	3.23	1.9	2.64	1.4	2.23	1.0	3.63	1.7	3.88	1.7
GeA	2.36	1.4	0.64	1.1	2.18	0.9	1.87	1.1	3.06	1.1	4.12	3.6	3.26	1.5	4.51	0.5	4.33	0.1	9.77	1.0
VA	4.04	1.3	3.20	1.2	3.37	1.2	3.08	1.1	4.43	1.3	3.91	2.3	3.03	1.3	2.78	0.7	4.16	1.7	4.47	1.8
SyrA	4.39	1.3	3.36	1.3	3.72	1.2	5.01	1.1	5.07	1.3	4.38	2.2	3.28	1.3	3.02	0.4	4.61	1.7	5.01	3.7

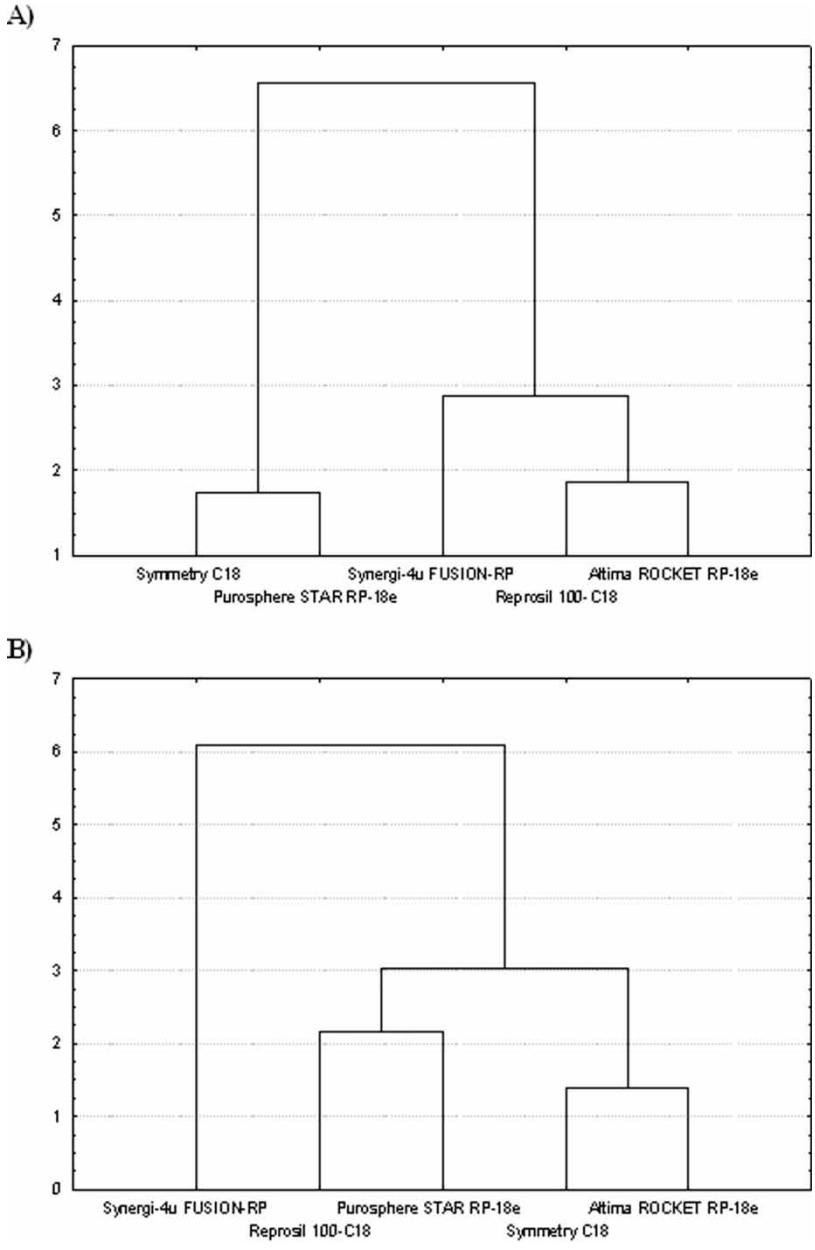


Figure 3. The partition of columns used in the study on the basis of cluster analysis for: A) pH = 3; B) pH = 5.8.

group in the stationary phase ligand. Synergi-FUSION-RP is not end-capped, it uses dual phase selectivity: a polar group is embedded in a hydrophobic ligand to achieve improved selectivity. Hydrogen bonds participate in the benzoic acid retention on this column at higher pH, which decreases the peak symmetry and increases the analysis time.

Four octadecyl packings create the second cluster, among which two groups are characteristic. The largest similarities at pH = 5.8 are typical for Symmetry C18 and Alltima ROCKET RP-18e. According to pK_a values listed in Table 2, the benzoic acids derivatives are partially dissociated around pH = 4.0. When the pH is above 5.8, carboxylic groups of studied compounds are totally ionized, therefore, compounds are negatively charged. The same situation is characteristic also for residual silanols, which became negatively charged with the increase of mobile phase pH. This is why the retention of analyzed compounds decreased with the changed pH from 3.0 to 5.8. In the retention mechanism of benzoic acids, repulsion forces between negatively charged substances and silanols started to play a significant role. Assigning the Symmetry C18 and Alltima ROCKET RP-18e to one cluster and the Purosphere STAR RP-18e, Reprosil 100-C18 to the other cluster is a consequence of different silanol activity of different stationary phases. It is probably caused by different ways of end-capping or by surface heterogeneity of these stationary phases.

CONCLUDING REMARKS

There is no correlation between obtaining similar results on different octadecyl columns. Each column from a distinct manufacturer (even if it is the same octadecyl type one) often gives different analysis results. The use of chromatographic methods for column evaluation allow the determination of similar or dissimilar column packing materials. The examination of benzoic acids derivatives analysis allowed for qualification of differences in chromatographic columns and also provided an explanation of distinctive reasons. Although all four octadecyl stationary phases were octadecyl, end-capped there were observed differences in performance of the used stationary phases. Those distinctions are a consequence of packings surface heterogeneity, and with the use of statistical approaches results in the possibility of column partition with regard to different analyzed compounds retention at pH = 3.0 and pH =5.8.

ABBREVIATIONS

GA	gallic acid
PA	protocatechic acid
pHBA	p-hydroxybenzoic acid

VA	vanillic acid
SyrA	syringic acid
GeA	gentisic acid
RP HPLC	reversed phase high performance liquid chromatography
CA	cluster analysis

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