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Classification and analysis of corn steep liquor by UPLC/Q-TOF MS and HPLC

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ABSTRACT

Corn steep liquor (CSL), an important raw material with high nutritional value, serves as a nitrogen source in the fermentation industry. The CSL quality directly affects the yield and quality of fermentation products. In this work, a fingerprinting technique was used to identify the potential markers of CSL. Forty-two CSL samples from different manufacturers were profiled by ultra-performance liquid chromatography with tandem quadrupole time-of-flight mass spectrometry. Sixteen compounds, almost all of which were amino acids and their derivatives, were considered as the potential markers. Then, *o*-phthalaldehyde-9-fluorenylmethyl chloroformate precolumn derivatization by high-performance liquid chromatography was performed to identify the free amino acids in CSL. Principal component analysis (PCA) was used to distinguish among the samples from different manufacturers. The results demonstrated that the fingerprinting technique combined with PCA analysis was a powerful tool for determining the CSL quality.

1. Introduction

The fermentation industry is rapidly developing [1–4]. In the wet-milling industry, corn is used to produce corn starch and the surplus raw material becomes corn steep liquor (CSL) by concentration.

CSL is used as a substrate material to produce glutamic acid, penicillin, lactic acid, hyaluronic acid, and others. CSL is also used as a nutritional and functional supplement to water-soluble plant proteins and vitamins during the fermentation process [5,6]. Soluble solids, which account for nearly 40–50% (w/w) of the dry weight of CSL, comprise a mixture of reducing sugars and amino acids, most of which are free amino acids. However, the chemical characterization of CSL is unclear. Currently, no method can reliably identify the key substance that distinguishes among CSL samples from different manufacturers. Nevertheless, a study determined the relationship between CSL quality and 2-keto-L-gulonic acid production using gas chromatography–mass spectrometry (GC–MS), atomic absorption spectrometry, and inductively coupled plasma-atomic emission spectroscopy (ICP-AES). A total of 17 marker compounds were found using GC–MS and ICP-AES [7].

Given the frequent usage of CSL, a new method of identifying marker compounds is necessary. Chemical fingerprinting is an internationally accepted, efficient technique for the quality control of complex analytes [8–12]. Metabonomics, a high-throughput chemical fingerprinting technique, can provide distinctive information [13–16]. Metabonomics uses multivariate statistical analysis to identify disease-related potential biomarkers and metabolic pathways. These strategies inspired our group to identify "potential markers" that distinguish among CSL from different manufacturers to evaluate the CSL quality.

In our prior studies [17,18], determination of main categories of components and four vitamins, including dry matter, total

Abbreviations: Ala, alanine; Arg, arginine; Asn, Asparagine; Asp, aspartic acid; CSL, corn steep liquor; Cys, cysteine; ESI-MS, electrospray ionization mass spectroscopy; 9-FMOC, 9-fluorenylmethyl chloroformate precolumn derivatization; GC–MS, gas chromatography–mass spectrometry; Glu, glutamate; Gly, glycine; HPLC, high-performance liquid chromatography; ICP-AES, inductively coupled plasma-atomic emission spectroscopy; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; OPA, *o*-phthalaldehyde; OPA-FMOC, *o*-phthalaldehyde 9-fluorenylmethyl chloroformate precolumn derivatization; PC, principal component; PCA, principal component analysis; Phe, phenylalanine; Pro, proline; Q-TOF MS, quadrupole time-of-flight mass spectrometry; Ser, serine; Thr, threonine; Try, tryptophan; Tyr, tyrosine; UPLC, ultra-performance liquid chromatography; Val, valine

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sugars, total reducing sugars, total free amino acids, total nitrogen, total sulfite and total acidity, were performed. Near-infrared spectroscopy was used to distinguish the raw materials obtained from different manufacturers. Samples from different companies were clustered. However, no study on the "potential markers" in CSL has been previously reported. Thus, ultra-performance liquid chromatography with tandem quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF MS) was used in the present study to acquire an initial profile of CSL and identify its "potential markers". The detected "potential markers" were almost all amino acids and their derivatives. Therefore, *o*-phthalaldehyde-9-fluorenylmethyl chloroformate precolumn derivatization (OPA-FMOC) by high-performance liquid chromatography (HPLC) was used to determine the free amino acids in CSL [19–22]. Principal component analysis (PCA) analysis was used to distinguish the products from different batches by sample clustering.

2. Materials and methods

2.1. CSL samples

Forty-two batches of CSL were collected from the provinces and territories of Shijiazhuang (two batches), Yishui (one batch), Pizhou (one batch), Qinhuangdao (ten batches), Shandong (six batches), Henan (eight batches), Julong (eight batches), and Hebei (six batches). All samples were refrigerated at 4 °C and stored at room temperature before measurements.

2.2. Reagents

HPLC-grade acetonitrile and methanol were purchased from Merck & Co., Ltd. (Darmstadt, Germany). *o*-Phthalaldehyde (OPA), 9-fluorenylmethyl chloroformate (9-FOMC), and mercaptopropionic acid were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Ultrapure water for samples and buffer solutions was prepared by a Milli-Q SP Reagent Water System (Millipore Corporation, MA, USA). All other reagents were analytical grade.

The following reference compounds were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA): aspartic acid (Asp), glutamate (Glu), asparagine (Asn), serine (Ser), glycine (Gly), threonine (Thr), cysteine (Cys), alanine (Ala), arginine (Arg), tyrosine (Tyr), methionine (Met), valine (Val), tryptophan (Try), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), lysine (Lys), and proline (Pro).

2.3. Preparation of standard and sample solutions

To determine the concentration of free amino acids, 200 μL of each CSL sample was diluted to 2 mL with ultrapure water and centrifuged for 10 min at 10,000 r min^{-1}. The upper phase was removed and twice the volume of methanol was added to deposit the proteins after the same centrifugation step described above. The last upper solution was then passed through a 0.45 μm syringe filter.

Each reference compound was accurately weighed, dissolved in 1% hydrochloric acid, and diluted to the appropriate concentration. A mixed standard solution was prepared in 1% hydrochloric acid. All solutions were stored in the refrigerator at 4 °C and allowed to warm to room temperature before analysis.

The OPA solution was prepared by mixing 40 mg of OPA, 3.5 mL of borate buffer (0.4 molL^{-1}, pH 10.4), 3.5 mL of acetonitrile, and 63 μL of mercaptopropionic acid. After mixing, the OPA solution was passed through a 0.45 μm syringe filter. The FMOC solution was prepared by mixing 25 mg of 9-FMOC with 5 mL of acetonitrile, and then passed through a 0.45 μm syringe filter.

2.4. UPLC/Q-TOF MS

The analysis was performed using a Waters Acquity^{TM} UPLC/Q-TOF MS instrument (Waters MS Technologies, Manchester, UK). Compounds were separated on a Waters Acquity UPLC BEH C_{18} column (2.1 mm × 100 mm, 1.7 μm particle) by gradient elution. The column temperatures were 30 °C, and the binary mobile phase was composed of phase A (water with 0.1% formic acid) and phase B (acetonitrile). The gradient programs used for separation were as follows: 0–2 min, 0.5–0.5% B; 2–7 min, 0.5–20% B; 7–8 min, 20–70% B; 8–10 min, 70–100% B; 10–11 min, 100–0.5% B; and 11–12 min, 0.5–0.5% B. The flow rate was 0.4 mL min^{-1} and the injection volume was 2 μL.

Electrospray ionization mass spectroscopy (ESI-MS) was used in both negative and positive ion modes. In the ESI-MS analysis, the capillary voltages were 3.0 kV for the positive mode and 2.5 kV for the negative mode. The cone voltage was 30 V and the extractor voltage was 3 V. The BF lens voltage was 1 V for the positive mode and 3 V for the negative mode. The nebulization gas flow was 800 L h^{-1} at 350 °C, the cone gas flow was 50 L h^{-1}, and the source temperature was 100 °C. The Q-TOF Premier acquisition rate was 0.1 s per scan with a 0.02 s interscan delay. The instrument was operated with the first resolving quadrupole in the widepass mode (50–1500 Da). A lockspray was used to ensure accuracy and reproducibility, and leucine enkephalinamide acetate was used as the lock mass ($[M+H]^+=555.2931$, $[M-H]^-=553.2775$) at a concentration of 200 pg μL^{-1}.

2.5. HPLC system

HPLC-diode array detection was used to determine the concentration of free amino acids. Analysis was carried out on an Agilent-1200 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, on-line degasser, auto-sampler, and column heater–cooler. Separation was performed on an Agilent SB-C_{18} column (250 mm × 4.6 mm, 5 μm; Agilent Technologies, Newport, DE, USA) with a C_{18} precolumn (4 mm × 5 mm). The column temperature was 40 °C and the split ratio was 3:1. Gradient elution of 0.2:3.5:1000 triethylamine/tetrahydrofuran/sodium acetate solution (A) and 200:400:400 sodium acetate solution/methanol/acetonitrile (B) was performed, with the concentration of sodium acetate solution being 0.02 molL^{-1}. Both pH values of the A and B phases were adjusted to 7.2 ± 0.05 by 2% acetic acid solution. The gradient program used for separation was as follows: 0–8 min, 5–19% B; 8–20 min, 19–35% B; 20–34 min, 35–60% B; and 34–40 min, 60–70% B. The detection wavelength was 338 nm. All determinations were operated under the automatic precolumn derivatization injection mode of Aglient-1200 HPLC.

2.6. Multivariate statistical analysis

To identify potential markers from a large amount of data of CSL from different manufacturers by UPLC/Q-TOF MS that was used to perform semi-quantitative analysis and identify "potential markers", multivariate pattern recognition methods were used to determine the characteristic components, which was similar to the metabolomic analysis. PCA was used to determine the characteristic components of the different CSL samples to provide the basis for distinguishing the sources [23–25]. UPLC/Q-TOF MS data were imported into Markerlynx, a built-in software in Masslynx (V4.1 SCN639, copyright by 2008 Waters Inc.). After this work, data from Markerlynx were directly imported into Simca-P software (version 11.5, Demo, Umetrics, Umea, Sweden) due to the compatibility of data formats between the two

software programs. The results provided the reliable data parameters of the raw materials for ensuring a smooth fermentation process.

PCA of the HPLC data was used to distinguish the principal components (PCs). In this process, the selection and pretreatment of data were critical factors. Making the indices dimensionless with methods such as standardization, equalization, etc. was generally necessary. The standardization maintained the relative stability of the original values. Data standardization was performed using SPSS 20.0 (SPSS Co. Ltd., Chicago, IL, USA).

3. Results and discussion

3.1. UPLC/Q-TOF MS analysis

MS data were obtained in both positive and negative ion modes. Most constituents exhibited their quasi-molecular ions $[M-H]^-$ in the negative ion mode and their $[M+H]^+$ ions in the positive ion mode. UPLC/Q-TOF MS methods provide high accuracy in the analysis of qualitative, quantitative, and structural information. Accurate molecular data of each potential marker were determined up to four decimal places using UPLC/Q-TOF MS. The data are presented in Table 1.

Multivariate data analysis is often applied in combination with fingerprinting. The peak areas of all chemical markers were put into the PCA by the Waters Markerlynx software to identify the potential markers. Twenty-one ion fragments (Fig. 1 and Table 1) may be the origin of discrimination. A longer distance of a data point from the origin indicated a greater contribution to significance in the loading plot (Fig. 1). From Table 1, all the "potential markers" were detected in the positive mode, while eleven ion fragments were deleted in the negative mode. Generally speaking, amino acids lost the carboxyl group easily in UPLC/Q-TOF MS. Table 1 shows that peak 12 was the fragment of peak 1 (Pro), peak 3 belonged to peak 4 (Phe), peak 19 was the fragment of peak 6 (Iso), peak 13 was a part of peak 8 (Val), whose mass spectral fragmentation regularities were in the same way—the parent ion lost the carboxyl group. And peaks 7 and 17 were Gly ethyl ester. Almost all components were amino acids, namely, Pro, l-Tyr, l-Leu-l-Lys, Phe, Ile, Met, and Val, etc. Overall, a total of 16 components can be used as potential markers. The potential markers were mainly amino acids, which play an important role in metabolism as basic nutritional components.

PCA analysis was used to distinguish the products made in different batches by clustering samples that were similar among the 42 qualified CSL samples from different manufacturers. These samples were well separated by the PCA method (Fig. 2). In the scatter plot, they were classified into five groups. The group of the same manufacturer was far from the other manufacturers, which demonstrated that the samples in this group significantly differed from those in the other groups.

3.2. HPLC determination

Given that almost all potential markers were amino acids and their derivatives, a method of OPA-FMOC by HPLC was used to determine the free amino acids in CSL. Mixed standard stock

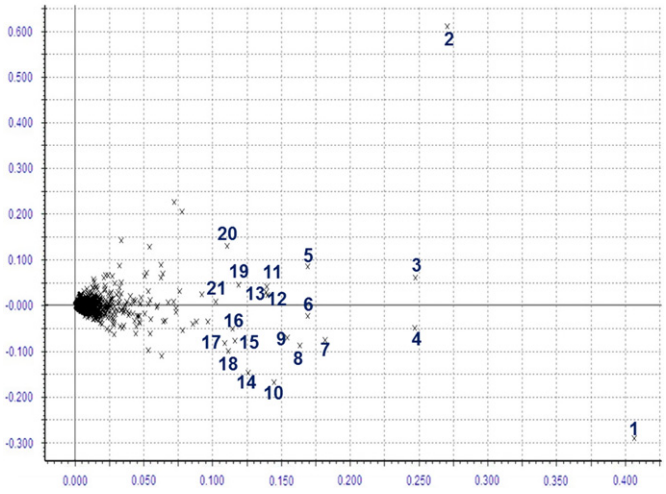

Fig. 1. X-loading score plot with 21 markers of CSL by UPLC/Q-TOF MS. The Waters Markerlynx software and SIMCA-P software (Version 11.5) were used.

Table 1
Potential markers of corn steep liquor by UPLC/Q-TOF MS.

No.	RT	Signification	Mode[a]	UV (nm)	Mass$[M+H]^+$/$[M-H]-$	Formula	Identification
1	0.69	0.3527	p	197	116.0708	$C_5H_9NO_2$	**Proline**
2	5.62	0.4729	p/ n	195	423.2571/421.2392	$C_{21}H_{34}N_4O_5$	L-Tyrosine, L-leucyl-L-lysyl-
3	3.68	0.1802	p/n	199	120.0767/118.0426	$C_9H_{11}NO_2$	**[Phenylalanine – COOH]**
4	3.68	0.1779	p/n	199	166.0861/164.0699	$C_9H_{11}NO_2$	**Phenylalanine**
5	1.32	0.1344	p/n	195	130.0862/128.0225	$C_6H_{11}NO_2$	**2-Amino-4-methylpent-4-enoic acid**
6	1.66	0.1204	p/n	195	132.1019/130.0740	$C_6H_{13}NO_2$	Isoleucine
7	0.62	0.1385	P	195	104.0706	$C_4H_9NO_2$	**Glycine ethyl ester**
8	0.88	0.1307	P	197	118.0864	$C_5H_{11}NO_2$	**Valine**
9	1.06	0.1193	P	194, 278	150.0594	$C_5H_{11}O_2NS$	Methionine
10	6.93	0.1564	p/n	245	493.2820/491.1047	$C_{23}H_{24}O_{12}$	Tricin-7-*O*-β-glucoside
11	0.73	0.1028	P	195	144.1027	$C_7H_{13}NO_2$	**2-Amino-4-methylhex-4-enoic acid**
12	0.69	0.1006	P	197	70.0654	$C_5H_9NO_2$	**[Proline–COOH]**
13	0.88	0.1000	P	197	72.0775	$C_5H_{11}NO_2$	**[Valine–COOH]**
14	3.47	0.1362	p/n	195	388.1197/386.1161	$C_{16}H_{21}NO_{10}$	4,7-Dimethoxy-2-((3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-2-yl)oxy)-2H-benzo[b][1,4]oxazin-3(4H)-one
15	5.51	0.0981	p/n	195, 220	371.1915/369.1721	$C_{15}H_{14}O_{11}$	2-*O*-Caffeoylhydroxycitric acid
16	8.61	0.0883	P	255	274.2743	$C_{16}H_{35}NO_2$	–
17	0.62	0.0960	P	195	104.0706	$C_4H_9NO_2$	**Glycine ethyl ester**
18	0.61	0.1055	p/n	199	175.1192/173.1030	$C_6H_{14}N_4O_2$	Arginine
19	1.64	0.0904	p/n	195	86.0932/84.0596	$C_6H_{13}NO_2$	**[Isoleucine–COOH]**
20	4.17	0.1203	P	194	466.2401	$C_{20}H_{31}N_7O_6$	L-Proline, L-alanyl-L-alanyl-L-histidyl-L-alanyl-
21	1.61	0.0726	p/n	195	138.0919/136.0687	$C_8H_{11}NO$	1-(4-Aminophenyl)ethanol

[a] Mode: positive and negative.

solutions containing 18 analytes were prepared and diluted to a series of appropriate concentrations for the construction of calibration curves. Six concentrations of each analyte were injected in triplicate. The calibration curves were constructed by plotting the peak area versus the concentration of each analyte. Intra- and inter-day precision, stability, repeatability, and recovery tests were carried out, and the outcomes were good. Fig. 3 shows the fingerprint of the mixed standard stock solution.

Table 2 shows the average measured values of all free amino acids and the standard deviation from the CSL samples (forty-one samples were measured because one sample was lost during the determination process). For the total free amino acids, a small difference was observed between different batches from different manufacturers. However, the differences among the species and contents of free amino acids of the different CSL samples, which were expressed as the coefficient of variation (CV), were significant. Some amino acids such as Thr were not detected in all manufacturers. The possible reason was the different production processes and/or raw material varieties. Several samples had big CV values, indicating that the quality of the source of CSL was still not well controlled. Generally, the differences among the samples from different manufacturers were rather significant than within the same manufacturer, which demonstrated that the CSL samples were purchased and/or produced in a relatively controlled level.

PCA was performed using 41 samples that were put into the dimensionality reduction analysis program of SPSS 20.0. This study aimed to explain the observed variables in terms of the minimum possible number of factors, which depended on the initial eigenvalues of the main components and its contribution ratio. The description of the original variables was obtained by the ignition solution of the PCA. The initial eigenvalues were important indicator factors. About 92.925% of the total variance was from the former four factors, which meant that a four-factor model can explain the proportion of the experimental data. PCs 1 and 2 also contained a large proportion of the sample information (cumulative amount = 82.446%).

The component score coefficient matrix reflected the contribution of the PCs, and the component score coefficient symbols indicated the effects of increased or decreased PC values by the substances. The extraction method involved PC scoring. The results showed that Ile, Asn, Met, Lys, and the total had significant positive correlation coefficients for PC1, which meant that the coefficients also increased as PC1 increased. Pro had a negative correlation with PC1, which indicated that PC1 decreased during prolonged storage. The other components had less effect on PC1. For PC2, Asp, Pro, and Try had positive correlation coefficients, whereas Val, Cys, and Tyr had negative correlation coefficients.

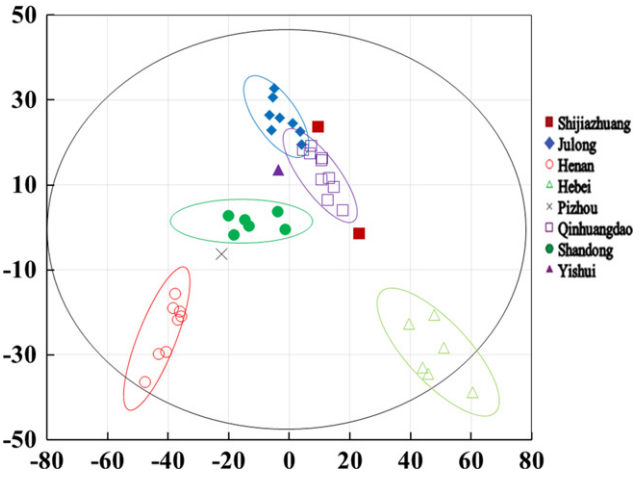

Fig. 2. 2D schematic of clustering of CSL by UPLC/Q-TOF MS.

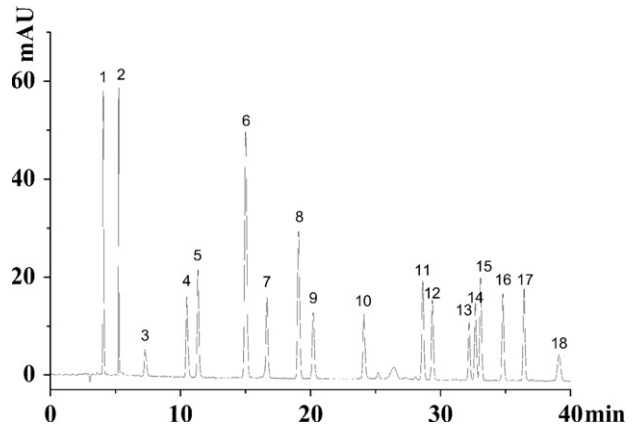

Fig. 3. HPLC fingerprint of mixed standard solution (1. Asp, 2. Glu, 3. Asn, 4. Ser, 5. Gly, 6. Thr, 7. Cys, 8. Ala, 9. Arg, 10. Tyr, 11. Met, 12. Val, 13. Try, 14. Phe, 15. Iso, 16. Leu, 17. Lys, and 18. Pro).

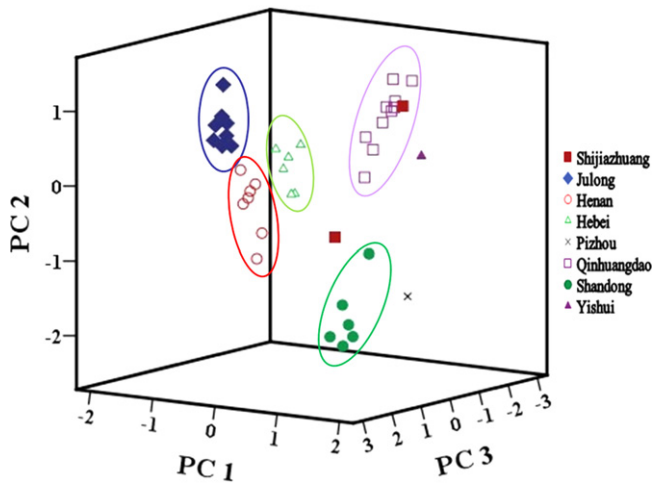

Fig. 4. 3D schematic of clustering of amino acids concentrations of CSL by HPLC. The SPSS 20.0 software was used.

Table 2
Determination of free amino acids in corn steep liquor by HPLC ($mgmL^{-1}$).

Batches	Asp	Glu	Asn	Ser	Gly	Cys	Ala	Arg	Tyr	Met	Val	Phe	Try	Ile	Leu	Lys	Pro	Total
Min	0.00	0.00	0.39	0.07	0.55	11.55	0.23	1.11	0.25	0.96	0.00	0.75	0.96	0.57	2.64	0.20	14.04	49.15
Max	3.07	2.32	1.73	2.57	2.06	36.08	2.49	7.87	2.07	3.30	1.00	2.10	3.51	1.61	9.47	2.64	31.28	90.63
Average	1.14	1.14	1.05	1.18	1.18	23.73	1.04	3.24	0.85	1.74	0.14	1.29	2.14	1.01	5.72	1.23	20.27	68.07
SD[a]	1.01	0.65	0.39	0.73	0.48	7.45	0.51	1.39	0.37	0.59	0.29	0.43	0.76	0.32	2.00	0.86	3.75	12.70
CV[b]	88	57	38	62	41	31	49	43	43	34	210	33	36	32	35	70	19	19

[a] SD, standard deviation.
[b] CV, coefficient of variation [(standard deviation/average) × 100].

For PC3, Val and Ala had positive correlation coefficients, whereas Arg had a significantly negative correlation coefficient. For PC4, Try and Val had positive and negative correlation coefficients, respectively.

Forty-one points from the samples were marked into the coordinate system to draw the 3D scatter plot, which included PC1 for the *X* axis, PC2 for the *Y* axis, and PC3 for the *Z* axis, as shown in Fig. 4. A good clustering effect was observed, which indicated that the differences among the CSL samples from different manufacturers were significant but those from the same manufacturer were not.

3.3. Comparison

A comparison of the two methods (PCA by UPLC/Q-TOF data and HPLC data) revealed consistent clustering results (Figs. 2 and 4). This finding indicated that the six amino acids, Ile, Asn, Met, Lys, Pro, and Asp, may be the characteristic components for identifying the different CSL sources.

The classification results indicated that this PCA model can be used for the quality control of CSL, and that CSL prepared by different pretreatment or manufacturing procedures and in different dosage forms have different qualities. The marker compounds were also affected by the corn origin, cultivation condition, harvest time, geographical climate, and environment. All these factors resulted in significant differences in the CSL quality.

4. Conclusion

The UPLC/Q-TOF MS method was used to obtain the mass spectra of the CSL samples. A fingerprinting technique was used to identify the potential markers. Sixteen components, almost all of which were amino acids, were eventually considered as the potential markers. Therefore, an OPA-FMOC method by HPLC was used to determine all free amino acids, and PCA was performed to distinguish the six PCs that were amino acids (Ile, Asn, Met, Lys, Pro, and Asp). This method was an easier method because ordinary apparatuses instead of advanced and expensive equipment were required to determine the stability of raw materials. This method can also be applied to other related fields.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.01.044.

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