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Development of 4-Chloro-3,5-dinitrobenzotrifluoride Derivatization and Ultrahigh-performance Liquid Chromatography Method for Determination of 20 Free Amino Acids in Mature Vinegar

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A rapid ultrahigh-performance liquid chromatography method has been established for the simultaneous determination of 20 free amino acids in mature vinegar. The chromatographic conditions, pretreatment methods and matrix effects were optimized. 4-Chloro-3,5-dinitrobenzotrifluoride was selected as a precolumn derivatizing reagent and can readily react with both primary and secondary amines. The separation of 20 amino acids was achieved within 15 min by gradient elution mode. The detection limits for the 20 different amino acids studied ranged from 1 to 9 mg L^{-1}. The correlation coefficient values ($r^2 = 0.9945$) indicated good correlations between the concentrations of the compounds under investigation and their peak areas within the responding ranges. Samples spiked at 50, 80 and 100 mg L^{-1} showed recoveries ranging from 70.1 to 109.2 % (except for tryptophan spiked with 50 mg L^{-1}, which showed a recovery of 59.3 %) with relative standard deviations below 5.6 %. The developed method was rapid and showed high levels of efficiency, accuracy and reproducibility that could be used effectively for the simultaneous determination of 20 free amino acids in mature vinegar.

Keywords: Amino Acids, Ultra high performance liquid chromatography, 4-Chloro-3,5-dinitrobenzotrifluoride, Mature vinegar.

INTRODUCTION

Amino acid molecules contain both amine and carboxylic acid functional groups and humans consume many foods that contain these amino acids. The analysis of amino acids is important for a wide range of application areas including foods, pharmaceuticals and variety of other biological applications, because these molecules play an important role in the nutritional quality of food and in the control of samples fortified with proteins[1,2].

Mature vinegar, which is inky black in color and possesses a complex malty flavor, is an aged fermented product that can be made from rice, wheat, millet, sorghum, or a combination of these ingredients. Traditionally, mature vinegar is more complex than European vinegar which is usually fermented from wine, cider, fruit juices and honey[3]. As a traditional condiment, mature vinegar has long been a commonly used flavoring in cooking and food processing. Although the main components of mature vinegar are water and acetic acid, other low-level components such as amino acids also have an important effect on mature vinegar products. Keeping this in mind, it is necessary to have a reliable method to determine the amino acids present in mature vinegar[4].

To date, several methods have been developed to separate and detect amino acids. Chemical derivatization is usually required for the effective detection of amino acids because most amino acids do not possess a strong chromophore or fluorophore and are therefore not readily detectable. For this reason, it is necessary to transform the analytes into derivatives that can be more readily separated and detected[5]. Traditionally, the determination of amino acids has been achieved by ion-exchange chromatography, followed by postcolumn derivatization with ninhydrin. However, this method requires dedicated equipment[6], In recent years, gas chromatography[7,8], capillary electrophoresis[9,10] and liquid chromatography[11,12] methods have been developed for amino acid determination. Of these methods, the use of reversed phase high performance liquid chromatography (HPLC) with precolumn derivatization has become a widely accepted technique. Compared with conventional liquid chromatography, ultrahigh-performance liquid chromatography (UHPLC) has increased the speed of analysis as well as the levels of resolution, sensitivity and peak capacity and has consequently become increasingly popular. Typical derivatizing reagents include phenyl isothiocyanate (PITC)[13] and 2,4-dinitrofluorobenzene (DNFB)[14], which are used in conjunction with UV detection, whereas *o*-phthaldialdehyde[15,16],

dansyl chloride[17], 9-fluorenylmethyl chloroformate[18] and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate[19] have been used in conjunction with fluorescence detection. Each of these reagents has its own specific advantages and limitations[20]. 4-Chloro-3,5-dinitrobenzotrifluoride shows strong UV absorption and can react readily with both primary and secondary amines. Derivatization with 4-chloro-3,5-dinitrobenzo-trifluoride proceeds rapidly at moderate temperatures, with the resulting derivatives being very stable and of high ultraviolet absorption. With the exception of the by-products resulting from the 4-chloro-3,5-dinitrobenzotrifluoride hydrolysis product, no multiply derivatized impurities or by-products are ever detected in the reaction mixtures during the 4-chloro-3,5-dinitrobenzotrifluoride derivatization process. The application of an excess of the reagent and the occurrence of its hydrolysis compound do not have any adverse impact on the separation process[1].

To the best of our knowledge, to date there have been no studies exploring the use of ultrahigh-performance liquid chromatography in conjunction with 4-chloro-3,5-dinitrobenzotrifluoride derivatization for amino acid determination. Although there have been several reports in the literature investigating the use of 4-chloro-3,5-dinitro-benzotrifluoride derivatization for amino acid determination. These reports typically used HPLC and required long analysis time. For example, Shi *et al.*[1] used HPLC with 4-chloro-3,5-dinitrobenzotrifluoride derivatization for the determination of amino acids in beer with an analysis time of 45 min. Based on this lack of UHPLC-based methodology in the literature for amino acid determination, we decided to develop an ultrahigh-performance liquid chromatography method using 4-chloro-3,5-dinitrobenzotrifluoride as precolumn derivatizing reagent with the aim of establishing a rapid, efficient and accurate method of amino acid determination. Furthermore, there have been few reports in the literature investigating the free amino acids in vinegar[21,22]. Especially the well-known mature vinegar of China. The objective of this work was to establish a rapid, efficient and accurate ultrahigh-performance liquid chromatography method based on 4-chloro-3,5-dinitrobenzotrifluoride precolumn derivatization for the simultaneous determination of 20 free amino acids in mature vinegar.

EXPERIMENTAL

The following 20 amino acids standards were supplied by RC Biological Science Co., Ltd. (Beijing, China), including aspartic acid (Asp), glutamic acid (Glu), aspargine (Asn), histidine (His), glutamine (Gln), serine (Ser), arginine (Arg), threonine (Thr), proline (Pro), glycine (Gly), alanine (Ala), valine (Val), methionine (Met), tryptophan (Trp), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), cystine (Cys2), tyrosine (Tyr) and lysine (Lys), with purities greater than 98 %. Stock standard solutions of the individual compounds with concentrations of 5000 mg L^{-1} (except for tyrosine and cystine with concentrations of 2000 and 800 mg L^{-1}) were prepared by the exact weighing of the powder into a 50 mL volumetric bottle followed by dissolution in 0.05 M hydrochloric acid (HCl) solution. The resulting solutions were then stored at 4 °C in brown bottles. A standard amino acid mix solution containing each of the individual compounds at a concentration of 250 mg L^{-1} (except cystine with a concentration of 100 mg L^{-1}) was prepared through the appropriate dilution of the stock solutions with 0.05 M HCl and subsequently stored at 4 °C in a brown bottle. HPLC-grade acetonitrile was supplied by Honeywell Burdick & Jackson (Muskegon, MI, USA). 4-Chloro-3,5-dinitrobenzotrifluoride (purity greater than 99.9 %) was obtained from T-Rich Co., Ltd. (Taiyuan, China). A 4-chloro-3,5-dinitrobenzotrifluoride solution was prepared in acetonitrile. A borate buffer (pH 9; H_3BO_3 - $Na_2B_4O_7$ buffer) was prepared by mixing a 0.2 M boric acid solution and a 0.05 M sodium tetraborate solution with the volume ratio of 1: 4. All of the other chemicals, including anhydrous sodium acetate, sodium tetraborate and boric acid, as well as the solvents, including glacial acetic acid, triethylamine and hydrochloric acid, were purchased as the analytical grades from Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Deionized water was purified using an Ulupure Ultra-pure water system (Chengdu, China).

A waters acquity UPLC system (Waters, Milford, PA, USA), consisting of a binary solvent manager, a sample manager fitted with a 10 µL loop and a diode array detector, was used for the analysis of the vinegar samples. Waters MassLynx 4.1 software (Waters) containing the Quanlynx program was used for chromatographic data processing. An acquity UPLC BEH C18 column (100 mm × 2.1 mm i.d., particle size 1.7 µm) purchased from Waters was used for the separation. An XW-80A vortex (Troody Technology Co., Ltd, Shanghai, China), WHY-2 thermostat water bath (Jincheng Guosheng Instrument Co., Ltd, Jiangsu, China) were used for derivatization. Millipore filtering apparatus (Millipore, Billerica, MA, USA) and filters of 0.45 µm pore size were used for the mobile phase.

Ultrahigh-performance liquid chromatography conditions: Prior to the UHPLC analysis, the C18 column, which was equipped with a guard column (4 mm × 3 mm i.d), was pre-equilibrated with the mobile phase for 0.5 h. Ultrahigh-performance liquid chromatography separation of the 4-chloro-3,5-dinitroben-zotrifluoride derivatives of the 20 amino acids was carried out on an Acquity UPLC BEH C18 column using gradient elution. A 20 mM NaAc solution containing 0.24 % triethylamine (v/v) and 0.13 % acetic acid (v/v) (eluent A) and acetonitrile (eluent B) were used as the mobile phases with a flow rate of 0.50 mL min^{-1}. The column temperature was kept at 40 °C and the injection volume was 10 µL. The following elution gradient was used. An initial mobile phase composition of 10 % eluent B was used that was subsequently increased to 25 % following a period of 5 min. This composition was then maintained for 2.5 min, before being increased to 55 % following 2.5 min and then increased to 65 % following a further 3 min. The mobile phase composition was finally returned to the initial conditions following 0.1 min and maintained at this composition for 1.9 min prior to the beginning of the next analysis, providing a total run time of 15 min.

Derivatization procedure: The derivatization procedure was established according to a relevant and recently published report[1]. A sample (200 µL) of the appropriate amino acid standard, the borate buffer (pH 9; 1 mL) and the 4-chloro-3,5-dinitrobenzotrifluoride solution (600 µL) were added

sequentially to a 10 mL vial. The resulting solution was then thoroughly mixed on a vortex mixer for 1 min and incubated at 60 °C for 0.5 h. Upon completion of the derivatization process, the resulting solution was diluted to 5 mL with borate buffer and stored at 4 °C. The derivative solution was then filtered through a 0.45 μm nylon filter membrane prior to analysis by UHPLC.

Statistical analysis: The data were subjected to paired sample T testing using minitab 15 (PA, USA) and were also subjected to analysis of variance (ANOVA) and Hierarchical cluster analysis using SPSS 16.0 (Chicago, IL, USA) software. All of the statistical analyses were performed in triplicate and at a confidence level of 95 %.

Analysis of real samples: The mature vinegar samples used for the study were purchased from a local supermarket and detailed information pertaining to their suppliers and compositions are shown in Table-4. The samples were diluted five times with deionized water and stored in glass bottles in a refrigerator at 4 °C. Their derivatization procedure was the same as that reported above for the standards.

RESULTS AND DISCUSSION

Optimization of the separation conditions: The chromatographic conditions used for the UHPLC analysis of the 20 derivatized amino acids were optimized to obtain the best peak shapes and reduce the overall analysis time. The column temperature was investigated in the first instance and found to have an obvious effect on the separation of proline and glycine. The method was therefore investigated at 30, 40 and 45 °C (Fig. 1). When a temperature of 30 or 40 °C was used, proline and glycine were successfully separated. Furthermore, the tryptophan and leucine provided higher levels of resolution at 40 °C. Based on these results, a temperature of 40 °C was selected for the column temperature. We then proceeded to optimize the gradient profiles and found that the separation of serine and arginine was obviously affected by the gradient profiles. Several gradient profiles were investigated and a good response was ultimately obtained using the gradient described in the experimental section (Fig. 2). Several other parameters were also investigated to provide a fast and reliable separation method, including the flow rate as well as the amounts of triethylamine and acetic acid included as additives. The results revealed that a flow rate of 0.50 mL min^{-1} was optimum together with the addition of 0.24 % (v/v) triethylamine and 0.13 % (v/v) acetic acid.

Selection of the reagent blank: Unfortunately the samples used for the method development were not standard reference materials and no blank mature vinegar samples were available. For this reason, 6 % (v/v) acetic acid, deionized water, 0.05 M hydrochloric acid and samples of the mature vinegar spiked with the amino acids at two different concentrations (50 and 80 mg L^{-1}) were processed according to the derivatization procedure and their recoveries were compared using the paired sample T test. For the spiked 6 % acetic acid sample, the recoveries of the majority of the amino acids were significantly different from the spiked mature vinegar sample ($P \geq 0.04$). The difference between them could be attributed to the carboxylic acid group in acetic acid, which may affect the reaction of the amino acids with the 4-chloro-3,5-dinitrobenzotrifluoride. For the deionized water and 0.05 M hydrochloric acid samples, the recoveries of the majority of the amino acids were not significantly different from those observed in the spiked mature vinegar sample ($P \geq 0.05$). Based on these results, 0.05 M hydrochloric acid was selected to investigate the LOD and LOQ values of both the instrument and method in the current study.

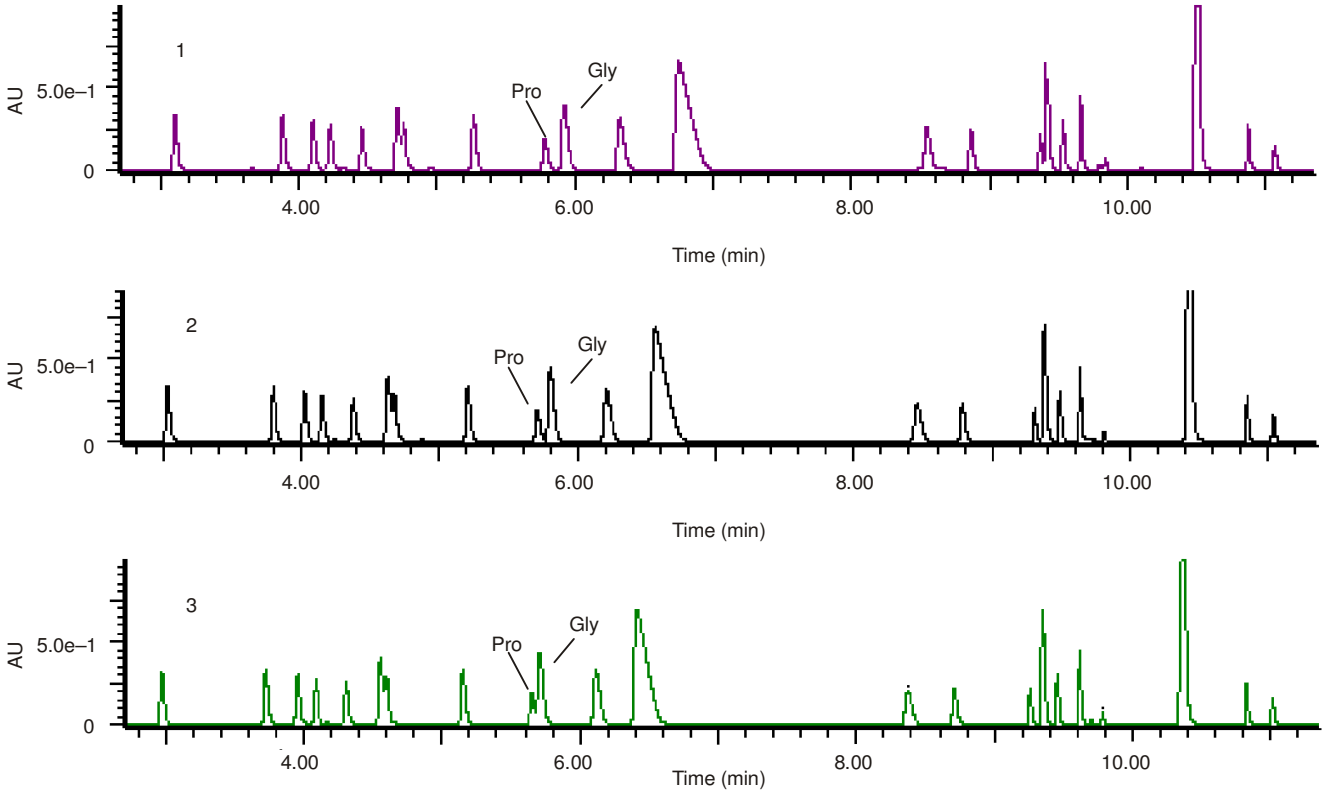

Fig. 1. Effect of column temperature on the separation of proline and glycine 1.30 °C, 2.40 °C, 3.45 °C

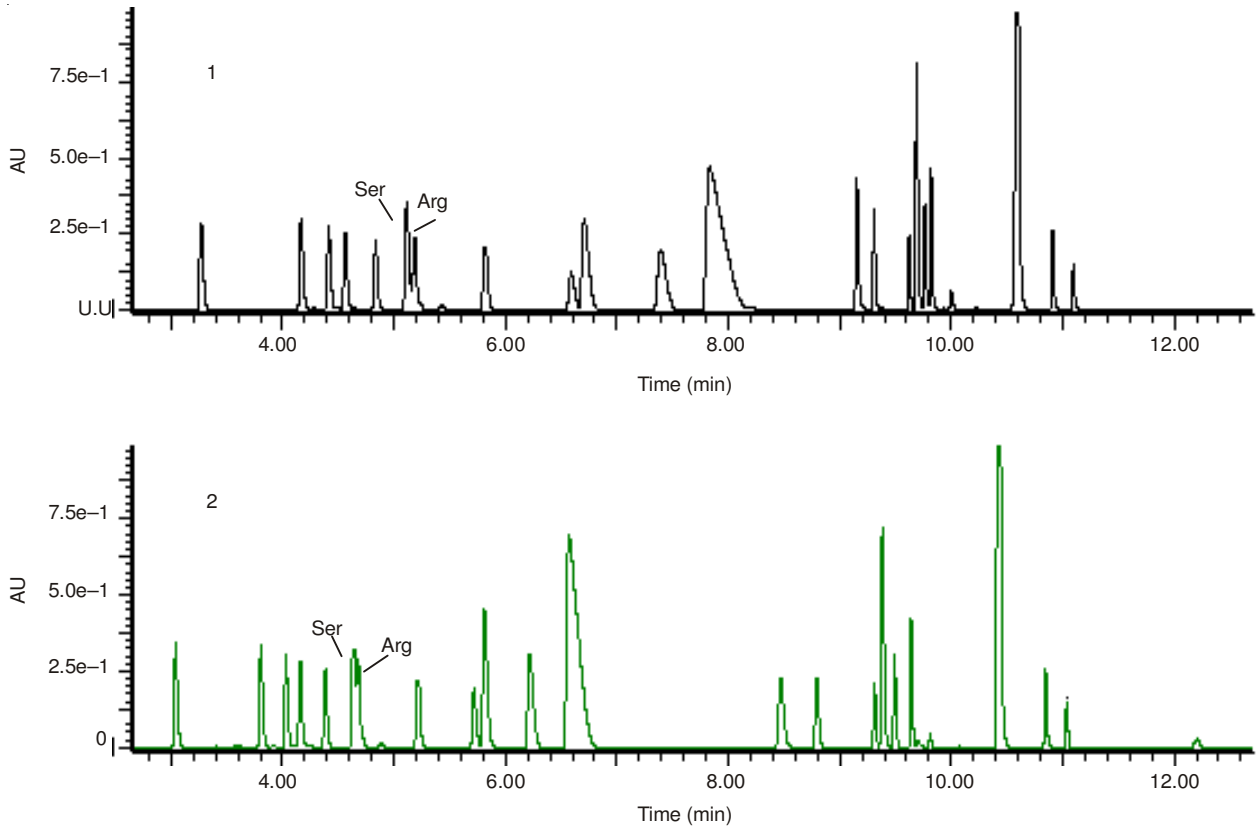

Fig. 2. Effect of gradient profiles on the separation of Ser and Arg 1. the optimized gradient profile 2. The initial gradient profile

Matrix effects: Based on the reference method[23], the matrix effect in the current study was investigated by comparing the calibration curves of the standard mixture of amino acids (10, 20, 50, 80, 100, 150, 200 and 250 mg L^{-1} for 19 amino acids; 20, 50, 80, 100, 200 and 500 mg L^{-1} for cystine) with vinegar by dissolving the samples in 0.05 M HCl and comparing them with a 5 % (v/v) mixture of 0.05 M HCl and vinegar. A paired sample T test was applied to compare the slopes of the different calibration curves. The results obtained revealed no significant difference between the two curves ($P \geq 0.08$). Based on these results, it was concluded that our new method for the determination of amino acids in mature vinegar was free from matrix interferences and the standard curve solutions were therefore prepared in 0.05 M HCl.

Optimization of the pretreatment for mature vinegar: The peak areas of 20 amino acids were compared following two different pretreatment methods. In the first of these pretreatment methods, no pretreatment was applied[21] to the mature vinegar, whereas the second pretreatment process involved passing the diluted samples through millipore filters with a 0.45 µm pore size[22]. The results subsequently obtained were compared by statistical analysis using the paired sample T test. The results suggested that no significant difference existed between the two pretreatment methods and the impact they had on the downstream analyses ($P \geq 0.06$). Based on these results, no pretreatment process was used in the current work.

Validation of the method: The amino acids in the samples were identified by comparison with the retention time of amino acid standards solutions. For the determination of retention time, the reference standards were injected both individually and as a mixture.

The limit of detection (LOD) and limit of quantification (LOQ) values were determined as the concentrations providing responses three (LOD) and ten (LOQ) times the average baseline noise with six replicate injections of the stock standard solution. These data are summarized in Table-1. The LOD values for the amino acids studied ranged from 1 valine to 9 mg L^{-1} lysine, whereas the LOQ values ranged from 3 valine to 30 mg L^{-1} lysine.

The linearity of the method was evaluated by injecting a series of amino acid samples of different concentrations, including 10, 20, 50, 80, 100, 150, 200 and 250 mg L^{-1} for 19 of the amino acids and 20, 50, 80, 100, 200 and 500 mg L^{-1} for cystine. These analyses were repeated six times at each of the concentration levels. The calibration curves were obtained using the external standardization method, in which the absolute peak areas were correlated to the concentration of the amino acid in question. The calibration functions of the compounds were linear and the determination coefficients were found to be greater than 0.9945 for all of the compounds (Table-1).

Recovery and reproducibility: The accuracy of the amino acid determination method was estimated through recovery studies. The recovery and reproducibility measures of the method were investigated by spiking the mature vinegar sample with standard analytes at three different concentration levels (50, 80 and 100 mg L^{-1}), with each analysis being repeated six times. At the same time, a mature vinegar sample without any spiking was also analyzed using the same method. In this way, the recovery could be calculated. Table-2 shows the results obtained from these experiments. It is clear from these data that the recoveries for the selected compounds ranged from 70.1 to 109.2 % (except tryptophan spiked with 50 mg L^{-1},

TABLE-1
LINEAR RANGE, CALIBRATION CURVES, CORRELATION COEFFICIENTS, LOD, LOQ OF TWENTY 4-CHLORO-3,5-DINITROBENZOTRIFLUORIDE-AMINO ACIDS DERIVATIVES (n = 6)

Analyte	Linear range (mg L^{-1})	Calibration curves	R^2	LOD (mg L^{-1})	LOQ (mg L^{-1})
Aspartic acid	10-250	y =106.771x - 16.548	0.9995	2.5	8
Glutamic acid	10-250	y = 106.955x - 247.388	0.9992	2.5	8
Aspargine	10-250	y = 88.600x - 240.061	0.9990	3.0	9
Histidine	10-250	y = 77.425x + 33.425	0.9962	3.0	9
Glutamine	10-250	y = 68.073x + 185.581	0.9990	3.5	10
Serine	10-250	y = 95.569x - 16.872	0.9945	3.5	10
Arginine	20-250	y = 67.743x - 61.235	0.9980	4.0	13
Threonine	10-250	y = 124.649x - 29.583	0.9990	3.0	9
Proline	20-250	y = 56.401x + 152.184	0.9987	4.5	15
Glycine	10-250	y = 166.728x + 245.510	0.9983	3.0	9
Alanine	10-250	y = 170.639x - 43.947	0.9995	2.5	8
Valine	10-250	y = 121.669x - 49.141	0.9995	1.0	3
Methionine	10-250	y = 89.937x + 252.307	0.9996	2.5	8
Tryptophan	10-250	y = 55.778x - 19.914	0.9988	3.5	10
Leucine and isoleucine	10-250	y = 202.430x - 33.270	0.9993	3.0	9
Phenylalanine	10-250	y = 71.767x - 11.364	0.9995	3.5	10
Cystine	20-500	y = 54.474x + 43.917	0.9989	4.0	13
Tyrosine	20-250	y = 76.806x - 58.493	0.9950	5.0	16
Lysine	50-250	y = 53.219x + 49.077	0.9958	9.0	30

x: concentrations (mg L^{-1}) of amino acids; *y*: peak area of amino acids derivatives

TABLE-2
INITIAL, ADDED AND DETERMINED CONCENTRATIONS (mg L^{-1}) WITH PRECISION (RSD/ %) AND MEAN RECOVERY (%) WITH PRECISION (RSD/ %) IN THREE FORTIFIED LEVELS (n = 6) FOR MATURE VINEGAR

Analyte	Initial	Added	Determined	Recovery	Analyte	Initial	Added	Determined	Recovery
Aspartic acid	23.1 (0.9)	50.0	58.7(0.9)	71.2(1.4)	Alanine	47.6 (1.4)	50	85.5(1.3)	76.2(1.3)
		80.0	80.6(1.3)	72.3(1.4)			80	109.8(1.5)	78.4 (1.3)
		100.0	106.2(1.8)	83.1(2.3)			100	132.2(2.7)	84.6(4.2)
Glutamic acid	69.2 (0.5)	50.0	109.2(1.2)	80.1(1.3)	Valine	25.9 (1.7)	50	62.3(1.2)	73.8(1.4)
		80.0	135.0(1.4)	82.6(1.7)			80	83.6(1.4)	72.4(1.4)
		100.0	142.8(2.0)	73.6(3.8)			100	113.1(2.6)	87.2(3.3)
Aspargine	-	50.0	48.5(0.8)	97.5(1.0)	Methionine	-	50	39.9(2.7)	80.0(2.5)
		80.0	76.6(1.5)	96.3(1.0)			80	73.1(1.8)	91.5(2.2)
		100.0	94.6(2.1)	94.7(2.2)			100	86.7(3.4)	86.7(3.4)
Histidine	3.4 (4.5)	50.0	50.8(1.8)	95.2(2.1)	Tryptophan	-	50	29.7(6.2)	59.3(6.8)
		80.0	79.3(1.7)	95.4(2.2)			80	70.7(4.1)	88.2(4.5)
		100.0	99.0(3.7)	95.6(3.8)			100	69.7(5.5)	70.1(5.5)
Glutamine	-	50.0	49.3(1.2)	99.0(1.0)	Leucine and isoleucine	21.0 (0.5)	50	59.2(1.4)	76.4(2.6)
		80.0	78.3(1.5)	98.1(2.0)			80	82.6(1.4)	77.5(1.3)
		100.0	96.8(2.5)	96.8(2.5)			100	111.7(2.6)	90.7 (3.3)
Serine	20.1 (3.1)	50.0	56.2(1.6)	72.4(1.4)	Phenylalanine	-	50	54.5(1.1)	109.2(0.9)
		80.0	84.0(1.5)	80.3(1.1)			80	80.8(1.4)	101.5(1.0)
		100.0	117.7(2.5)	97.6(3.1)			100	105.2(2.6)	105.2(2.6)
Arginine	9.7 (1.2)	50.0	50.7(2.7)	82.5(3.7)	Cystine	-	50	48.7(2.3)	97.3(2.1)
		80.0	76.2(3.6)	83.6(3.6)			80	79.7(1.1)	100.4(1.0)
		100.0	100.4(3.0)	90.7(3.4)			100	102.7(1.8)	102.7(1.8)
Threonine	14.5 (1.4)	50.0	57.5(1.4)	86.1(2.3)	Tyrosine	12.8 (5.0)	50	66.9(1.2)	108.6(1.9)
		80.0	83.2(1.4)	86.4(1.2)			80	96.7(2.6)	105.2(2.0)
		100.0	104.9(2.4)	90.4(2.7)			100	102.9(3.6)	90.1(4.1)
Proline	31.4 (1.5)	50.0	70.8(1.6)	79.5(2.5)	Lysine	20.2 (1.7)	50	63.3(3.2)	86.1(4.7)
		80.0	92.0(1.0)	76.8(1.0)			80	105.0(3.1)	106.3(3.8)
		100.0	117.4(2.3)	86.0(3.2)			100	117.3(4.6)	97.1(5.6)
Glycine	39.3 (0.8)	50.0	77.9(2.1)	79.1(2.6)					
		80.0	100.8(2.0)	77.5(1.3)					
		100.0	113.8(2.5)	74.5(3.8)					

- not detected

which provided a recovery of 59.3 %). Furthermore, the relative standard deviations were below 5.6 % in all cases. These results indicated that the developed method performed well and provided consistent and reproducible results.

Precisions: To determine intra-day and inter-day precisions for the analyses of amino acids in mature vinegar, three identical samples were analyzed on three separate days. The intra-day precisions for the individual measurements of the

TABLE-3
AVERAGE CONCENTRATIONS OF FREE AMINO ACIDS IN DIFFERENT COMMERCIAL MATURE VINEGAR SAMPLES (n = 3)

Analyte	Average concentrations (mg L^{-1}) and RSD (%) of free amino acids in different commercial mature vinegars									
	1#	2#	3#	4#	5#	6#	7#	8#	9#	10#
Aspartic acid	87.8a (3.6)	153.7d (3.6)	269.2gh (1.5)	197.2e (1.5)	276.2h (1.8)	165.3d (2.4)	131.8c (1.9)	115.5b (0.9)	258.0g (4.5)	221.3f (1.5)
Glutamic acid	285.0b (6.3)	425.3d (3.2)	444.2d (5.2)	478.8e (3.6)	604.0f (0.5)	262.5b (3.5)	149.0a (3.4)	346.0c (0.5)	491.5e (3.7)	352.0c (4.5)
Arginine	Δ	Δ	73.7c (7.2)	60.0b (4.6)	45.3a (2.3)	Δ	Δ	-	67.7bc (5.6)	73.2c (5.1)
Histidine	32.7a (6.2)	-	112.7c (5.0)	120.8c (5.0)	72.3b (5.2)	Δ	-	Δ	158.0d (4.5)	116.8c (3.9)
Glutamine	-	-	-	-	-	-	-	-	-	-
Serine	180.8c (1.8)	187.7c (3.6)	284.3e (2.1)	302.2f (1.9)	424.5g (1.3)	214.0d (3.1)	147.5b (1.2)	100.5a (3.1)	574.2i (1.5)	500.2h (3.5)
Arginine	152.3bc (0.9)	144.8b (2.4)	285.0d (2.4)	289.0d (3.0)	284.2d (1.5)	161.0c (3.0)	108.0a (2.4)	Δ	390.7f (2.8)	330.0e (3.3)
Threonine	119.8c (1.2)	93.3b (5.1)	173.2d (2.6)	191.0e (3.9)	220.7f (2.7)	112.3c (3.5)	75.5a (5.2)	72.3a (1.4)	363.5g (1.3)	356.5g (3.3)
Proline	244.3c (0.6)	223.0c (3.3)	309.3e (2.8)	360.7f (3.1)	515.5g (1.8)	268.8d (2.5)	186.8b (1.3)	156.8a (1.5)	861.5i (2.6)	771.3h (3.5)
Glycine	379.2c (2.1)	226.3a (4.4)	408.5c (2.8)	529.0d (2.9)	544.5d (1.2)	297.2b (2.3)	223.3a (1.3)	196.5a (0.8)	1307.2e (1.2)	1399.2f (3.7)
Alanine	700.5d (1.4)	413.2b (3.6)	948.5e (2.0)	1035.2f (2.1)	1194.5g (1.1)	560.2c (2.1)	560.2c (2.3)	238.2a (1.4)	2177.2h (1.7)	2147.8h (3.1)
Valine	218.0c (1.6)	161.2b (3.6)	376.7d (2.9)	377.3d (5.3)	428.2e (1.0)	193.3c (3.4)	119.0a (0.1)	129.7a (1.7)	823.8g (3.4)	751.2f (3.6)
Methionine	41.2a (4.8)	Δ	55.7ab (1.0)	60.7b (6.0)	76.5c (1.3)	43.3a (5.1)	Δ	-	67.5bc (4.6)	89.3d (4.9)
Tryptophan	-	Δ	Δ	-	-	-	-	-	-	-
Leucine and isoleucine	322.8d (1.0)	222.0c (4.6)	536.3e (2.7)	566.7f (3.0)	703.0g (1.2)	301.2d (3.0)	169.2b (2.2)	105.0a (0.5)	976.0i (1.7)	906.7h (3.4)
Phenylalanine	92.8b (1.6)	87.2b (3.7)	170.8d (2.8)	199.2e (3.0)	233.5f (1.4)	121.2c (2.3)	78.2a (1.5)	-	269.0g (2.7)	227.2f (3.5)
Cystine	88.7b (1.3)	81.2a (2.5)	-	-	-	-	-	-	-	-
Tyrosine	97.5c (3.6)	163.2d (7.4)	178.8d (3.1)	199.5e (4.3)	454.2g (1.0)	167.8d (2.0)	79.3b (1.6)	63.8a (5.0)	312.5f (3.1)	168.2d (2.7)
Lysine	168.3b (7.9)	183.7b (5.4)	377.0d (4.7)	407.7d (3.5)	548.7e (1.9)	212.8c (2.3)	112.8a (3.0)	101.0a (1.7)	644.5f (1.5)	387.5d (4.0)
Total essential*	963.0d (0.8)	747.3c (3.3)	1689.7e (2.6)	1802.5f (3.4)	2210.5g (1.1)	984.2d (2.5)	554.7b (5.1)	408.0a (1.2)	3144.3i (1.5)	2718.3h (3.5)
Total	3211.8d (0.9)	2765.7c (3.2)	5003.8e (2.4)	5374.8f (3.2)	6625.7g (0.7)	3081.0d (1.2)	2140.6b (4.2)	1625.3a (0.9)	9742.7i (0.7)	8798.3h (2.4)

- not detected; Δ below the limit of quantitation; *Sum of Threonine, Valine, Methionine, Tryptophan, Leucine, Leucine, Phenylalanine and Lysine
Means followed by different letters in the same row are significantly different from each other at $\rho < 0.05$ level

amino acids ranged from 0.5 to 5 %, whereas the inter-day precisions ranged from 4 to 9.8 % (RSD). Based on these results, it was concluded that the method provided good levels of precision.

Analysis of the commercial mature vinegar samples: The current method was used to determine the presence of free amino acids in different mature vinegars. With the exception of leucine and isoleucine, the amino acids were separated and quantified. Fig. 3 shows the chromatograms of a blank, a sample of the 100 mg L^{-1} mixed standard amino acid solution and a sample (mature vinegar 8#) spiked with a sample of the 100 mg L^{-1} mixed standard solution. The concentrations of the individual amino acids as well as the sums of the total essential and total amino acids in the different mature vinegars are shown in Table-3. The results revealed that the amounts of the total essential and total amino acids in product 9# from manufacture E were the highest, whereas the amounts in product 8# from manufacture D were the lowest. Glutamine was the only amino acid not to be detected in any of the samples. Tryptophan was only detected in product 2# from manufacture B and product 3# from manufacture C, *albeit* with a lower quantification limit. cystine was only found in product 1# from manufacture A and product 2# from manufacture B. Alanine was identified as the most abundant free amino acid in the majority of the samples, with the exception of product 2# from manufacture B and product 8# from manufacture D, where glutamic acid was the most abundant free amino acid in both cases. Asn was identified as the least abundant free amino acid in all of the samples, with the exception of product 3# from manufacture C and product 9# from manufacture E, where methionine was the least abundant free amino acid in both cases.

To evaluate variations in the different mature vinegars, ANOVA and HCA analyses were performed based on the indivi-

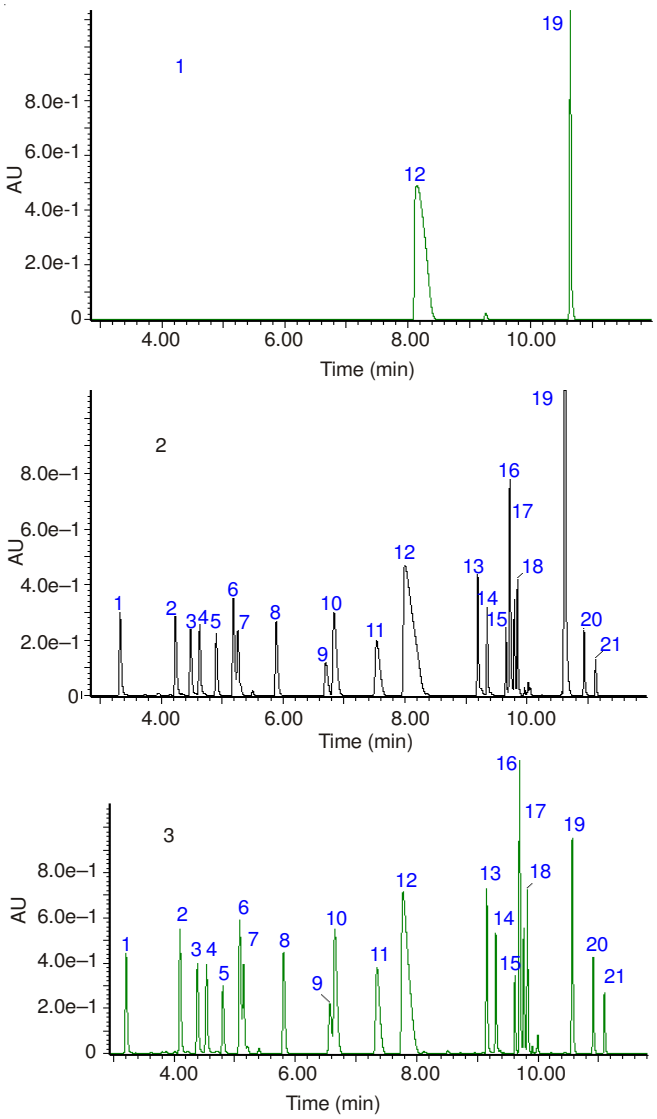

Fig. 3. Chromatograms of, 20 amino acids and CNBF derivatives 1. blank 2. 100 mg L^{-1} mixed standard amino acids 3. spiked samples with 100 mg L^{-1} mixed standards 1. aspartic acid 2. glutamic acid 3. aspargine 4. histidine 5. glutamine 6. serine 7. arginine 8. threonine 9. proline 10. glycine 11. glycine 12. (CNBF)OH 13. valine 14. methionine 15. tryptophan 16. leucine and isoleucine 17. phenylalanine 18. cystine 19. CNBF 20. tyrosine 21. lysine

dual, total essential and total amino acids contents of the samples. The ANOVA results are shown in Table-3. It is clear from the results that the individual, total essential and total amino acid concentrations in the different mature vinegar samples were significantly different from each other. The HCA results are shown in Fig. 4. The results revealed that 10 mature vinegars investigated could be divided into two main clusters (I and II) according to their contents. Samples 1# to 8# were in cluster I, which was divided into two subgroups (A and B), whereas samples 9# and 10# were in cluster II. The results indicated that the samples collected from the same manufacturer were predominately classified in one cluster. For example, products 3# and 4# from manufacture C were classified in the same cluster, whereas products 6#, 7# and 8# from manufacture D were classified in another cluster, together with products 9# and 10#, which implied that the manufacturers themselves exerted a significant influence over the contents of the 20 amino acids in the mature vinegars.

TABLE-4
INFORMATION OF ALL KINDS VINEGARS MENTIONED IN TABLE-3

Product code	Volume (mL)	Manufacture code	Production date
1#	800	A	December 8, 2011
2#	800	B	November 21, 2011
3#	800	C	December 1, 2011
4#	850	C	November 27, 2011
5#	820	D	December 16, 2011
6#	1000	D	December 7, 2011
7#	1750	D	October 28, 2011
8#	820	D	December 7, 2011
9#	1800	E	November 19, 2011
10#	2200	E	October 24, 2011

Conclusion

In conclusion, we have developed a rapid UHPLC method for the simultaneous determination of 20 free amino acids in mature vinegar using 4-chloro-3,5-dinitrobenzotrifluoride as a precolumn derivatizing reagent. The precolumn 4-chloro-3,5-dinitrobenzotrifluoride derivatization proceeded rapidly at ambient temperature, producing stable derivatives with high

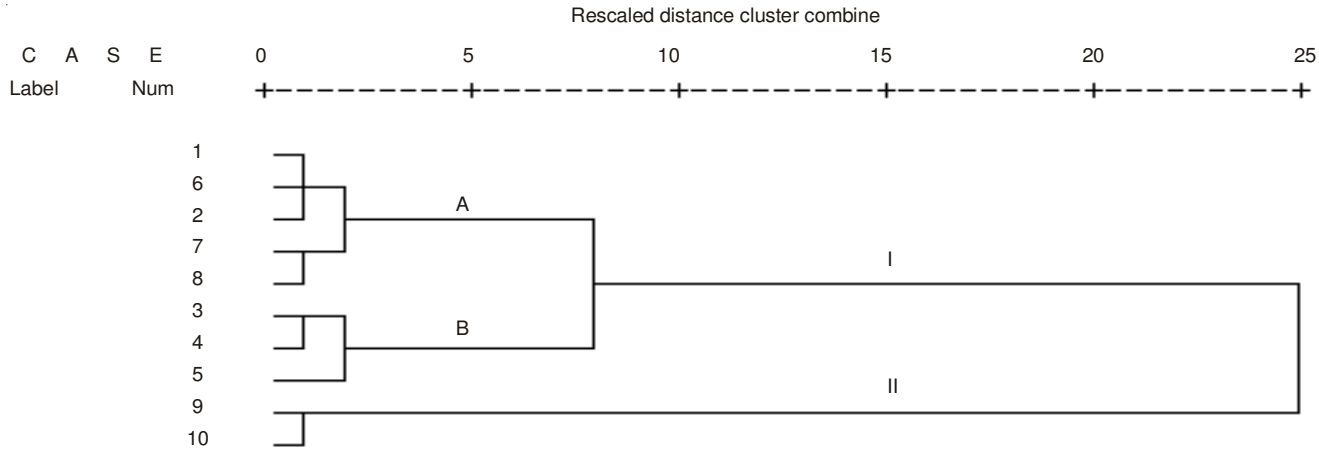

Fig. 4. Dendrograms of hierarchical cluster analysis for 10 mature vinegars in Table-4

levels of ultraviolet absorption. With the exception of the 4-chloro-3,5-dinitrobenzotrifluoride hydrolysis compounds, no multiple derivatives and by-products were observed in the reaction. Furthermore, the use of an excess of the 4-chloro-3,5-dinitrobenzotrifluoride reagent and its hydrolysis compound had no impact on the separation process. The simplicity of the 4-chloro-3,5-dinitrobenzotrifluoride technique combined with the high speed, resolution and sensitivity of ultrahigh-performance liquid chromatography provided a rapid, sensitive and accurate method for the simultaneous determination of 20 free amino acids in mature vinegar. Unfortunately, however, the separation of leucin and isoleucine was not possible with this method and further investigation is required.

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