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应用纪要

Simultaneous quantification of DL-amino acids in tea using a robust and sensitive LC-MS/MS method

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Abstract

Free amino acids (FAAs) are active compounds in tea which affect the qualities of taste, aroma, and color of the tea. FAA content plays a key role in determining the quality of the tea and is associated with the selling price, with some teas valued up to tens of thousands of dollars per kilogram. D-amino acids are known to be produced under food processing conditions such as fermentation, high temperature or strong acid or alkali treatments. Chiral FAAs may indicate the process and/or storage conditions for the tea product and could be used to grade and price tea through an analytical approach. This application note demonstrates a robust and sensitive UPLC™-MS/MS quantification method for chiral DL-amino acids in tea brew using the Xevo™ TQ-S micro Triple Quadrupole Mass Spectrometer. The analytical method exhibited good linearity for all amino acids, with R^2 >0.9934 and residual % <20 %. All enantiomeric pairs were well-resolved on the Daicel ChiralPax ZWIX+ column. With the current method, we were able to detect various D-form amino acids in oolong tea brew, which were on average 10,000-fold lower in concentration as compared to the L-form amino acids.

Benefits

- A robust and sensitive analytical method was developed to accurately quantify 37 DL-amino acids in tea samples using the ACQUITY UPLC I-Class PLUS coupled with the Xevo TQ-S micro Triple Quadrupole Mass Spectrometer
- Separation of all enantiomeric pairs of amino acid were easily achievable on the Daicel ChiralPax ZWIX+ column. This allowed selective and specific identification with accurate quantitation of each isomeric pair with identical MRM transitions
- ACQ derivatization has been proven to improve resolution of DL-amino acids, and boost signal intensity by five to 100-folds compared to underivatized method

Introduction

Tea is a widely consumed beverage and has many important physiological properties and potential health benefits. Free amino acids (FAAs) are key chemicals related to the taste and bioactive quality of tea brew.[1] Proteinogenic amino acids, except glycine, form a pair of enantiomers with L- and D-configuration. D- amino acids are naturally present at trace levels and are found in various fermented foods such as vinegar, beer, wine, and tea. In addition, fermentation, high temperature, strong acid, or alkali treatments induce the formation of D-amino acids.[3] The study of chiral FAAs may indicate the process and/or storage conditions for the tea product and could be used to grade and price tea through an analytical approach.

FAAs are highly polar with low volatilization and no chromophore, thus the simultaneous determination of FFAs acids in complex commodities, such as tea, is challenging. There are two common analytical approaches based upon high-performance liquid chromatography (HPLC) separation; determination in their native form or in a derivatized form where chemical groups are added, either pre- or post-column, to assist in the analysis. For the determination of FAA enantiomers, a variety of chromatographic methods using chiral derivatizing reagents, chiral mobile phases and chiral stationary phases have been reported. Those chiral columns that can be used with mobile phase systems compatible with mass spectrometry to separate derivatized FFAs, are extremely valuable for the determination of multiple FAAs, including those deficient of chromophores for UV detection. While specific types of derivatizations have been developed for chiral analysis, standard derivatization methods such as aminoquinolyl-N-hydroxysuccinimidyl carbamate (ACQ) have been utilized with chiral columns to allow for enantiomer

separation of FFAs. AQC has several advantages as compared with other derivatization reagents, including a simple and well-established derivatization procedure, a shorter derivatization time, fewer side reactions and excellent stability.[2] While UV and fluorescence detection are commonly used with ACQ derivatization, tandem quadrupole mass spectrometry (MS/MS) is preferred for the analysis of complex matrices due to its excellent selectivity. Tandem mass spectrometry only requires FAA enantiomers of the same mass (constitutional isomers) and same product ions to be chromatographically separated since overlap of amino acids with different masses does not affect determination.

In this application note, a robust and sensitive method was developed for the quantitation of 37 DL-amino acids in oolong tea brew using an ACQUITY™ UPLC I-Class PLUS System coupled with Xevo TQ-S micro tandem quadrupole mass spectrometer.

Experimental

Calibration Curve Standard Preparation

18 pairs of DL-amino acids and L-glycine were individually dissolved in deionized water (DI) water to a stock concentration of 100 µg/mL each. 2.5 µg/mL chiral amino acids standard mixture was prepared using methanol as diluent. The chiral amino acids mixture was derivatized using AccQ-Tag™ Ultra (p/n: 186003836 <https://www.waters.com/nextgen/global/shop/application-kits/186003836-accq-tag-ultra-derivatization-kit.html>) according to the protocol in the AccQ-Tag Ultra Derivatization Kit care and use manual.[4] Calibration curves were prepared by serial dilution using methanol to 0.25, 1, 2.5, 10, 25, 100, and 250 ng/mL respectively. A set of 25 ng/mL underivatized chiral amino acids standards mixture was also prepared using methanol as diluent.

Tea Sample Preparation

1 g of dry Wenshan Baozhong Oolong tea was weighed and brewed in 50 mL of 100 °C water for ten minutes. The supernatant was filtered, prior to derivatization with AccQ-Tag Ultra. The derivatized tea sample was diluted 10-fold to quantify L-amino acids and the undiluted derivatized tea sample was used to quantify D-amino acids.

Chromatographic Conditions

LC system:	ACQUITY UPLC I-Class PLUS
Column(s):	Daicel ChiralPak ZWIX+, 3.0 x 150 mm, 3 µm
Column temperature:	40 °C
Sample temperature:	10 °C
Injection volume:	5 µL
Flow rate:	0.150 mL/min
Mobile phase A:	25 mM Ammonium Formate + 0.1% Formic Acid in Methanol: Water (98:2)
Mobile phase B:	25 mM Ammonium Formate + 0.1% Formic Acid in Water

Gradient Table

Time (min)	%A	%B	Curve
Initial	100	0	Initial
45.0	80	20	6
45.1	100	0	6
55	100	0	6

MS Conditions

MS system:	Xevo TQ-S micro

Ionization mode:	ESI+
Desolvation temperature:	500 °C
Desolvation gas flow (L/Hr):	650
Cone gas flow (L/Hr):	150
Source temperature:	120 °C
Cone voltage:	30 V
Capillary voltage:	2 kV

Data Management

Chromatographic software:	MassLynx™ V4.2
MS software:	Masslynx V4.2
Quantitation software:	TargetLynx™

MRM Transitions for Derivatized Amino Acids

Same parameters were used to determine both the derivatized and underivatized amino acids standards and samples. Data was collected using MRM mode, with two transitions for each derivatized chiral amino acid. The MRM transitions and their respective collision energies were optimized using IntelliStart. The selected transitions were the same as those reported in literature.[1] Transitions given in bold are the quantifiers in Table 1. During optimization, we observed that derivatized arginine and theanine have identical quantifier transitions. Leucine and isoleucine also have identical MRM transitions as they are structural isomers.

S/N	Analytes		Parent *m/z*	Daughter *m/z*	CE (v)
1	DL-Alanine	Ala	260.2	**171.1**	20
2	DL-Arginine	Arg	345.2	**171.1**	20
			345.2	174.9	30
3	DL-Aspartic acid	Asp	304.2	**171.1**	20
			304.2	115.8	50
4	DL-Cystine	Cys	581.1	**171.1**	30
			581.1	411.2	20
5	DL-Glutamic acid	Glu	318.1	**171.1**	20
			318.1	116.0	50
6	L-Glycine	Gly	246.2	**171.1**	20
			246.2	115.9	50
7	DL-Histidine	His	326.2	**171.1**	10
			326.2	156	25
8	DL-Isoleucine	Ile	302.2	**171.1**	20
			302.2	116.1	50
9	DL-Leucine	Leu	302.2	**171.1**	20
			302.2	116.1	50
10	DL-Lysine	Lys	487.1	**171.1**	25
			481.1	145.2	25
11	DL-Methionine	Met	320.2	**171.1**	20
			320.2	116.1	50
12	DL-Phenylalanine	Phe	336.2	**171.1**	25
			336.2	116.1	50
13	DL-Proline	Pro	286.2	**171.1**	20
			286.2	115.9	30
14	DL-Serine	Ser	276.2	**171.1**	20
			276.2	116.1	50
15	DL-Theanine	Thea	345.1	**171.1**	40
			345.1	145.1	20
16	DL-Threonine	Thr	290.2	**171.1**	20
			290.2	115.8	50
17	DL-Tryptophan	Try	375.2	**171.1**	25
			375.2	188.1	20
18	DL-Tyrosine	Tyr	352.2	**171.1**	25
			352.2	116.0	50
19	DL-Valine	Val	288.2	**171.1**	20
			288.2	116.1	50

Table 1. MRM transitions of 37 chiral amino acids.

Results and Discussion

Eighteen pairs of derivatized DL-amino acids together with L-glycine were well separated on the Daicel ChiralPak ZWIX+ column using a 55-minute run time. Analytes with identical quantifier transitions such as isoleucine, leucine, arginine and theanine were resolved. Figure 1 shows the separation of individual DL-amino acid pairs.

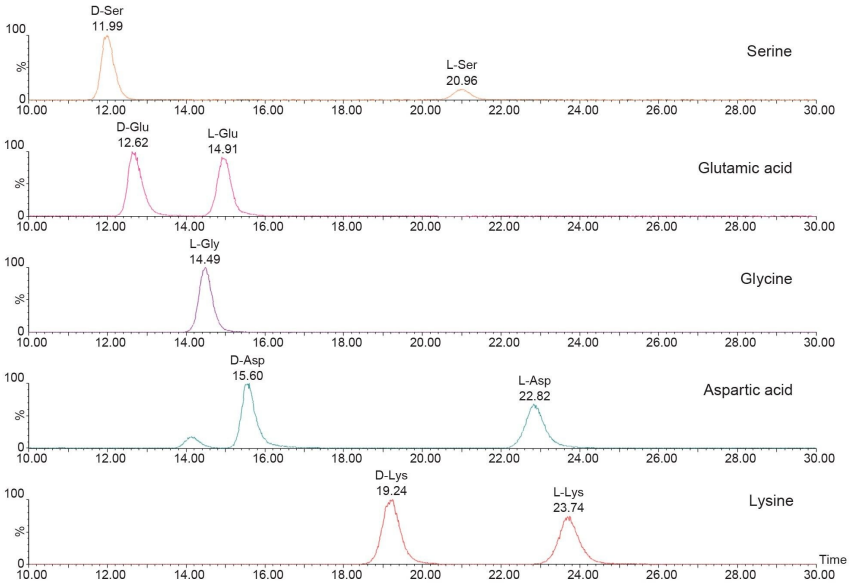

Figure 1. Chromatogram separation of a selection of individual DL-amino acid pairs at 25 ng/mL.

Instrument Linearity, Accuracy, Precision and Sensitivity

The linear dynamic range for all DL-amino acids is shown in Table 2. The coefficient of determination (R^2) achieved for all analytes was greater than 0.993, with residuals <20%. This shows excellent instrument linearity and accuracy for the quantitation of DL-amino acid on the ACQUITY I-Class PLUS System coupled with the Xevo TQ-S micro tandem quadrupole mass spectrometer.

Instrument precision was calculated using the data from six repeated injections at 1 ng/mL. The precision (%RSD) for retention time and peak area at 1 ng/mL was <15%, demonstrating good instrument precision

at low concentration. Instrument performance is summarized in Table 2 and 3.

Compound name	RT	R^2	Cal range (ng/mL)	S/N at lowest cal range
D-Alanine	9.95	0.997	0.25–100	174
D-Arginine	29.36	0.993	1–100	19
D-Aspartic acid	15.60	0.994	0.25–100	31
D-Cystine	47.09	0.999	1–250	22
D-Glutamic acid	12.62	0.999	0.25–100	64
D-Histidine	24.40	0.997	1–250	32
D-Leucine	7.82	0.995	0.25–100	126
D-Lysine	19.24	1.000	0.25–100	76
D-Methionine	11.66	0.997	0.25–100	138
D-Phenylalanine	10.90	0.995	0.25–250	79
D-Proline	9.15	0.999	1–100	23
D-Serine	11.99	0.998	0.25–250	28
D-Theanine	11.20	0.995	0.25–100	54
D-Threonine	9.96	0.997	0.25–250	52
D-Tryptophan	15.65	0.999	0.25–250	56
D-Tyrosine	11.91	0.997	0.25–250	68
D-Valine	7.95	0.998	0.25–100	176
L-Alanine	10.88	0.998	0.25–100	167
L-Arginine	48.83	0.999	1.00–100	12
L-Aspartic acid	22.82	0.998	0.25–100	24
L-Cystine	27.97	0.997	2.5–250	10
L-Glutamic acid	14.91	0.995	0.25–100	39
L-Glycine	14.49	0.998	0.25–250	62
L-Histidine	39.65	0.999	1–250	26
L-Isoleucine	9.50	0.996	0.25–100	86
L-Leucine/D-Isoleucine	8.94	0.995	0.25–100	171
L-Lysine	23.74	0.999	0.25–100	53
L-Methionine	13.40	1.000	0.25–250	43
L-Phenylalanine	13.19	0.995	0.25–100	66
L-Proline	8.48	0.998	1–100	21
L-Serine	20.96	0.999	0.25–250	21
L-Theanine	13.81	1.000	0.25–250	50
L-Threonine	17.09	0.998	0.25–250	14
L-Tryptophan	50.20	0.998	0.25–250	14
L-Tyrosine	16.19	0.999	0.25–250	50
L-Valine	9.12	1.000	0.25–100	157

Table 2. Details on instrument linearity and sensitivity for DL-amino acids.

	Reproducibility at 1 ng/mL (n = 6)			
Compound name	**Avg. RT**	**% RSD**	**Avg. area**	**% RSD**
D-Alanine	9.92	0.05	121457	1.53
D-Arginine	29.5	0.18	9875	12.58
D-Aspartic acid	15.59	0.13	44289	5.31
D-Cystine	47.13	0.21	14561	5.90
D-Glutamic acid	12.66	0.20	61981	3.39
D-Histidine	24.67	0.24	8441	7.89
D-Leucine	7.79	0.06	154173	1.40
D-Lysine	19.35	0.25	52763	4.45
D-Methionine	11.66	0.11	173159	1.36
D-Phenylalanine	10.79	0.15	103853	1.18
D-Proline	9.13	0.12	211675	5.11
D-Serine	11.95	0.09	57232	3.22
D-Theanine	11.20	0.10	53429	2.91
D-Threonine	9.92	0.12	114564	2.17
D-Tryptophan	15.58	0.05	66689	3.02
D-Tyrosine	11.92	0.05	57896	1.35
D-Valine	7.91	0.11	145453	2.12
L-Alanine	10.81	0.10	116786	1.53
L-Arginine	48.94	0.10	6075.4	6.30
L-Aspartic acid	23.01	0.13	32616	6.01
L-Cystine	28.39	0.89	15103	4.04
L-Glutamic acid	14.94	0.15	49287	3.01
L-Glycine	14.47	0.09	100571	2.08
L-Histidine	39.85	0.16	7212.6	2.15
L-Isoleucine	9.46	0.10	61127	4.60
L-Leucine/D-Isoleucine	8.86	0.12	212412	1.96
L-Lysine	23.89	0.14	44493	4.37
L-Methionine	13.40	0.20	53628	4.86
L-Phenylalanine	13.20	0.07	99420	2.22
L-Proline	8.41	0.21	144406	3.93
L-Serine	21.21	0.13	44905	3.07
L-Theanine	13.77	0.09	44480	4.06
L-Threonine	17.22	0.3	32336	4.28
L-Tryptophan	50.57	0.12	37951	7.15
L-Tyrosine	16.22	0.14	54638	2.51
L-Valine	9.09	0.12	129423	1.90

Table 3. DL-amino acid reproducibility at 1 ng/mL.

Free L-amino acids were determined in the oolong tea sample after brewing, dilution, and derivatization. The amount of L-amino acids present ranged from 3 to 3000 µg/g in the dry sample, after factoring the dilution due to infusion of the tea leaves. Since the D-form amino acids are reported to be present at trace levels in tea samples, undiluted derivatized oolong tea brew was used for their quantification. Twelve D-amino acids were detected in oolong tea sample, at concentrations ranging from 0.2 to 18.2 µg/g in dry sample. The FFA content for oolong tea brew is summarized in Table 4, and some chromatograms of DL-amino acids detected in tea brew are shown in Figure 2. The % D calculated

from our method coincide with literature studying D-amino acid profiles in different types of tea.[5] On average, the D-form amino acids were approximately 1,000-fold lower in concentration as compared to L-forms.

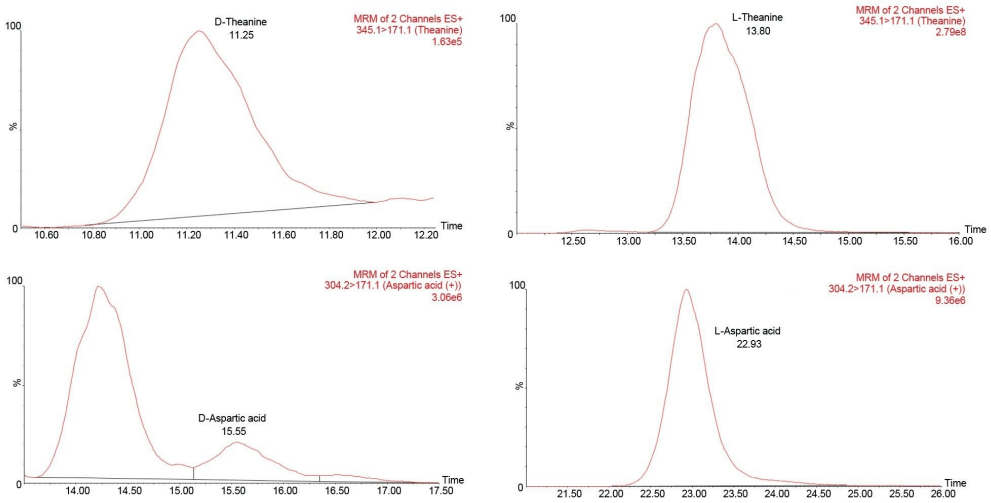

Figure 2. Selected chromatograms of DL-amino acids in oolong tea brew.

Name	L-form amino acid in dry sample (µg/g)	L-form amino acid in tea infusion (ng/mL)	D-form amino acid in dry sample (µg/g)	D-form amino acid in tea infusion (ng/mL)	% D (D/D+L)
Alanine	314	6280	N.D.	N.D.	N.D.
Arginine	130	2600	17	340	10.9
Aspartic acid	1020	20390	2.9	58	0.3
Cystine	N.D.	N.D.	N.D.	N.D.	N.D.
Glutamic acid	993	19860	18	364	1.8
Glycine	21	410	N.D.	N.D.	N.D.
Histidine	42	830	3.9	78	8.6
Leucine/Isoleucine	85	1690	0.3	5	0.3
Lysine	76	1510	N.D.	N.D.	N.D.
Methionine	3	60	0.2	4	6.2
Phenylalanine	141	2810	1.3	26	0.9
Proline	111	2220	N.D.	N.D.	N.D.
Serine	534	10670	2.1	42	0.4
Theanine	3213	64250	3.6	72	0.1
Threonine	396	7910	2.6	52	0.6
Tryptophan	247	4930	0.5	10	0.2
Tyrosine	118	2360	0.5	9	0.4
Valine	141	2820	N.D.	N.D.	N.D.

Table 4. Concentration of DL-amino acids in oolong tea brew.

Conclusion

Free D-amino acids are naturally present at trace levels in fermented foods such as tea. Different processing conditions affect the content and level of D-amino acids. It is important to have accurate determination of % D content in tea samples due to their direct impact on the product selling price. An MRM method for the determination of 37 DL-amino acids was successfully developed on the ACQUITY I-Class PLUS System coupled with the Xevo TQ-S micro tandem quadrupole mass spectrometer. Chromatographic separation of D- and L- amino acids, after derivatization, was achieved using the Daicel ChiralPax ZWIX+ column. Excellent instrument accuracy, precision and linear dynamic range for DL-amino acids were established in this study. The instrument was also capable of achieving LODs of 0.25 ng/mL for all amino acids except for L-arginine and L-cystine (1 ng/mL). The wide dynamic range of Xevo TQ-S micro allows for direct injection with minimal tea sample preparation to determine D/L amino acid ratio. The method uses a simple and quick approach to sample preparation that reduces the time and cost of the analysis, making this analytical method, established on an ACQUITY I-Class System, fitted with a Daicel chiral column, coupled to a Xevo TQ-S micro tandem quadrupole mass spectrometer,

attractive for routine commercial use to determine the D/L amino acid ratio of tea.

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