中国烟草总公司 郑州烟草研究院
ZHENGZHOU TOBACCO RESEARCH INSTITUTE OF CNTC

Simultaneous determination of 22 Amadori Compounds in tobacco leaves by LC-MS/MS

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Report Contnts

1 Research meaning

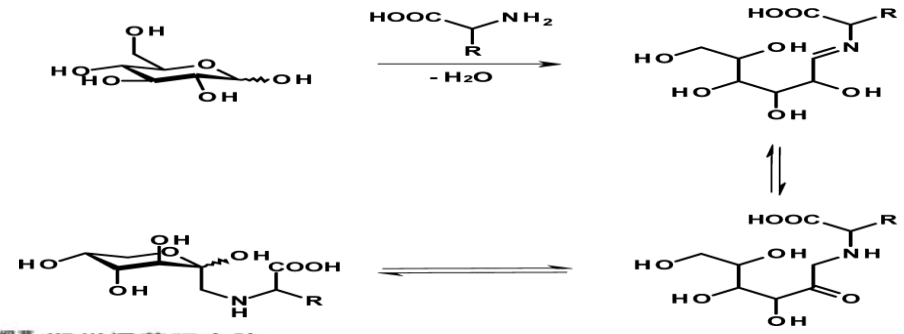
2 Research contents

3 Conclusion

Why will we determine Amadori compounds?

- Maillard reaction
  - The no enzymatic interaction **between reducing sugars and amino acids**, is one of the most important reactions in food and tobacco.
  - Maillard reaction is important to **the flavor and odor** of tobacco.
- Amadori Compounds

  Aroma precursors formed in the initial phase of the Maillard reaction by Amadori rearrangement of the corresponding glycosylamine.



Path way leading to the formation of Amadori reaction products from glucose and α -aminoacids.

Why will we determine Amadori compounds?

- The flue-cured tobacco contains large amounts of sugar (about 20% ~ 30%) and amino acid (about 1%), which provide the precursors of Maillard reactions.
- Curing, aging and processing of tobacco provide the reaction conditions of Maillard reactions.

Identifying and monitoring Amadori compounds is meaningful and necessary.

The reported quantification methods

- The earliest work focusing on HPLC-based procedures always involved post-column or pre-column derivatization with UV or fluorescence detector.

  Disadvantage: low sensitivity, selectivity

- GC has rarely been used.

  Disadvantage: complex derivatization procedure ;

  more than 2 or 3 peaks for each Amadori compound

- After 2000, LC-MS/MS became the main research direction to determine Amadori compounds based on its high sensitivity and resolution.

The reported quantification methods

Publication	Equipment	Derivative reagent	Column	Targets	Sample Matrix
Noguchi et al. Agri. & Biol. Chem.,1971, 35(1): 65.	Amino acid analyzer	Ninhydrine; post-column reaction	Shim-pack Amino-Na	6	Tobacco
Ian G.M. Anderson et al. British American Tobacco,1989: 1633.	HPLC-UV/VIS	TTC post-column reaction	Beckman DEAE silica.	6	Tobacco
R. H. Wang et al. Chinese J. Chromatogr. 2013, 31(12): 1189.	HPLC-MS/MS	-	Waters XBridgeTM Amide	6	Tobacco
L. Y. Liu et al. J. Sep. Sci. 2017, 40: 849–857	**HPLC-MS/MS**	-	**Atlantis T3 column**	**10**	**Tobacco**

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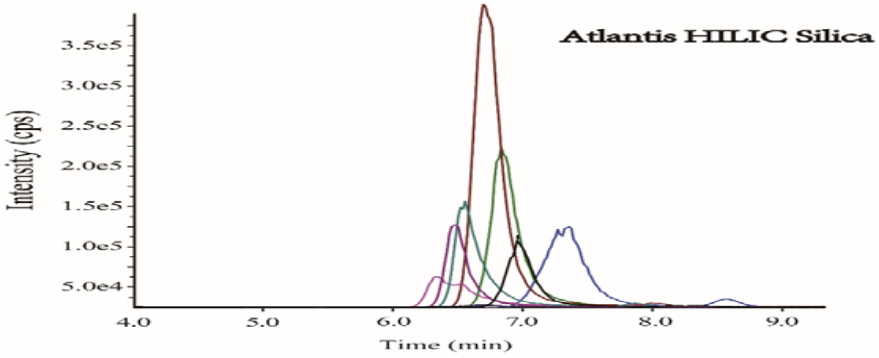
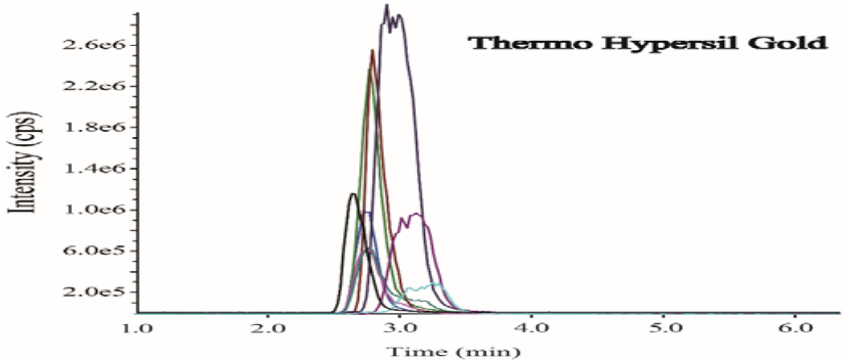
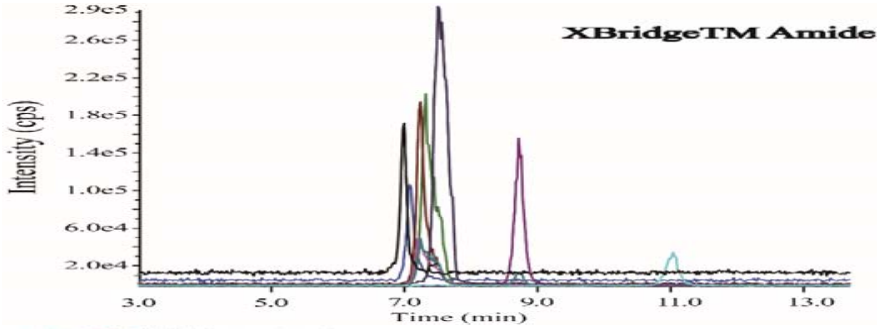
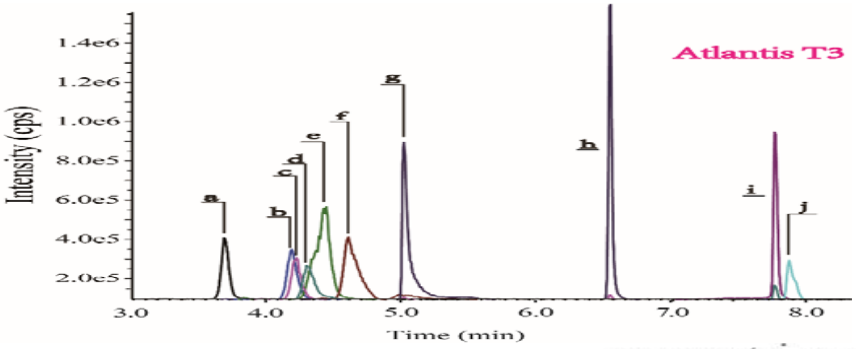
1) Determination of 10 Amadori Compounds by LC-MS/MS

Selection of column









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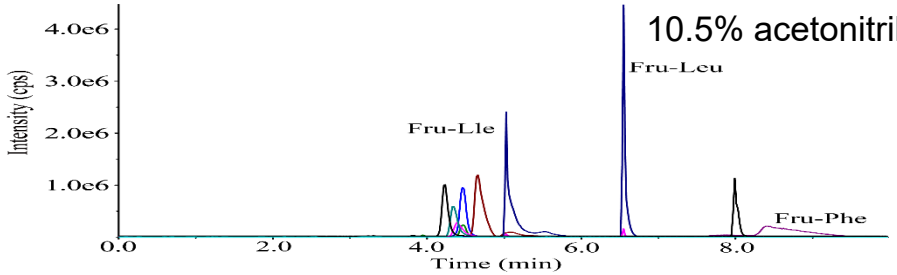
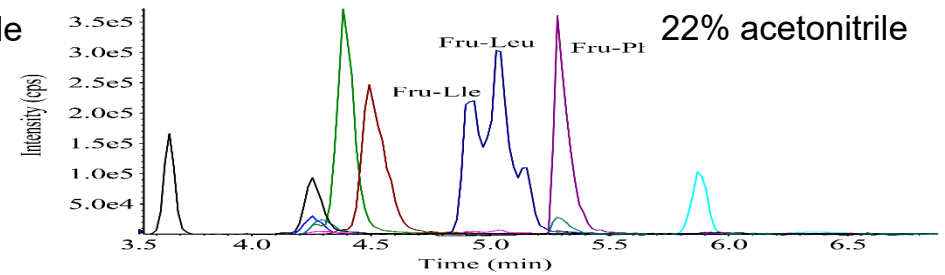
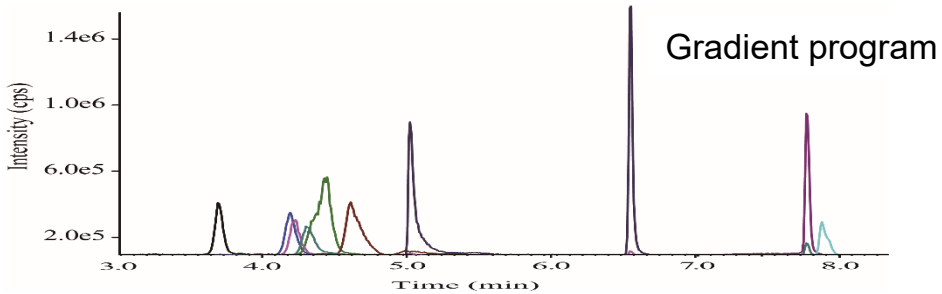
1) Determination of 10 Amadori Compounds by LC-MS/MS

Gradient elution







Time /min	Flow rate μL/min	0.2%formic acid	Acetonitrile
0.00	600	89.5%	10.5%
0.10	600	78%	22%
10.00	600	78%	22%

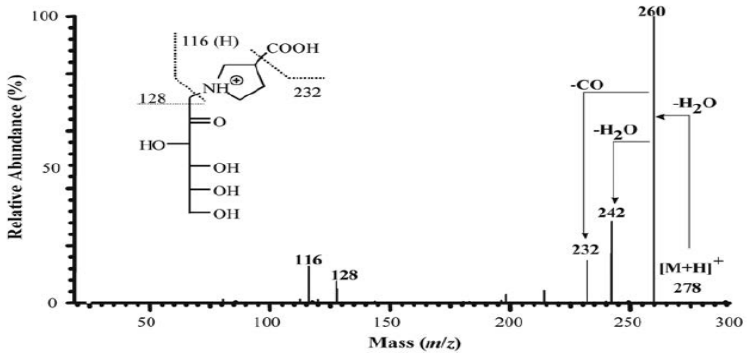
Column：Atlantis T3 (2.1×250mm, 5μm)，injection volume：5μL

1) Determination of 10 Amadori Compounds by LC-MS/MS

API 5500 mass spectrometer with an ESI source (ESI+)

Optimization of MS parameters

- ✓Temperature of turbo ion-spray source: 550 ° C
- ✓Ion-spray voltage: 5500 V
- ✓Curtain gas (N2) : 20 psi
- ✓Ion source nebulizer gas : 60 psi
- ✓ion source heater gas : 75 psi
- ✓collision gas : medium pressure
- ✓Detection mode: MRM



MS/MS spectrum of [M + H]+ ion of Fru-Pro at a collision voltage of 15 V

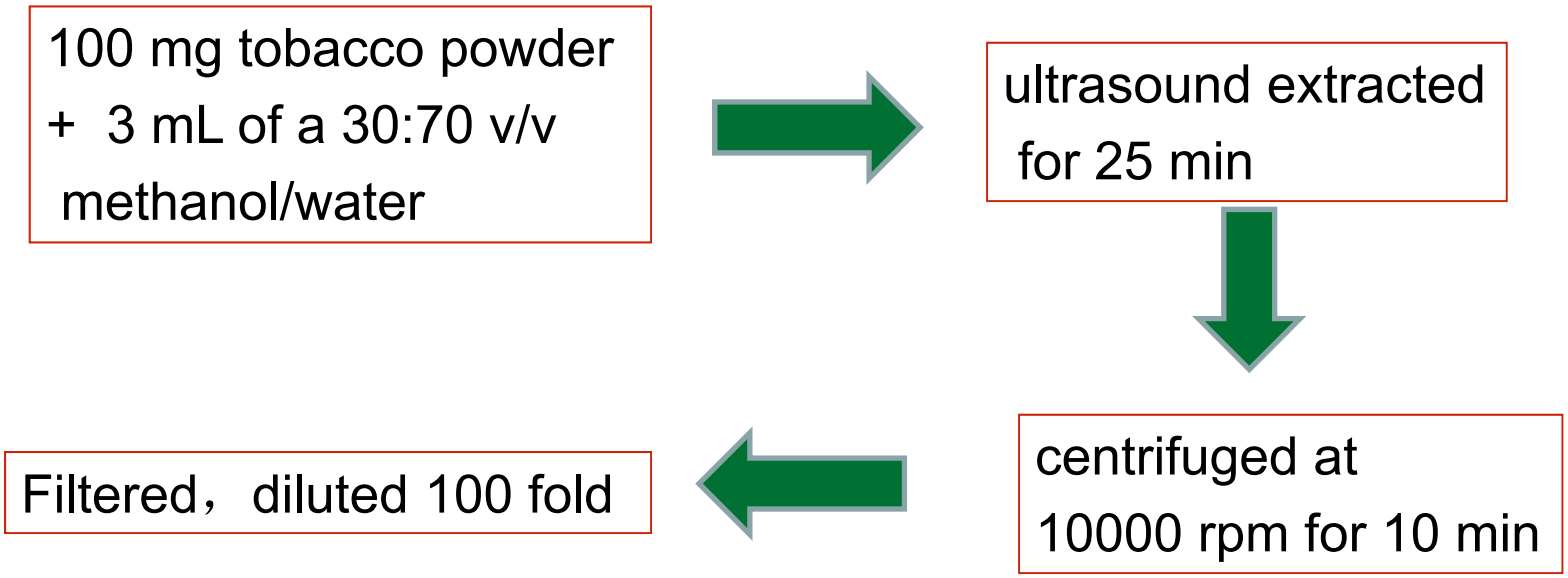
1) Determination of 10 Amadori Compounds by LC-MS/MS

MS/MS spectral data of [M+H]+, optimized declustering potential and collision energy

Compound	Precursor ion [M+H]+ (m/z)	Ion pair (m/z)	DP (V)	CE (V)
Fru-Leu	294.3	294.3/276.3*	52	13
		294.3/258.3	52	24
Fru-Asn	295.3	295.3/277.1	50	14
		295.3/259.3*	50	15
Fru-Trp	367.3	367.3/349.2*	57	15
		367.3/331.2	57	16
Fru-Pro	278.4	278.4/260.2*	41	15
		278.4/242.2	41	22
Fru-Phe	328.3	328.3/310.3*	41	13
		328.3/292.2	41	19
Fru-Val	280.3	280.3/262.2*	50	14
		280.3/244.2	50	21
Fru-Lle	294.3	294.3/276.3*	52	13
		294.3/258.3	52	24
Fru-Ala	252.3	252.3/234.1*	41	11
		252.3/216.1	41	19
Fru-Glu	310.3	310.3/292.1*	49	14
		310.3/274.3	49	18
Glucosamine	180.2	180.2/162.2*	48	11
		180.2/144.2	48	16

1) Determination of 10 Amadori Compounds by LC-MS/MS

Sample preparation



1) Determination of 10 Amadori Compounds by LC-MS/MS

External standard absolute quantification

Compound	Linear equation	r	Linear range, μg/mL	LOD, ng/mL	LOQ, ng/mL
Fru-Leu	$y=7.47\times10^3x+8.3\times10^3$	0.9990	0.005–1.00	1.568	5.229
Fru-Asn	$y=533x-1.44\times10^3$	0.9998	0.050–10.0	12.66	42.22
Fru-Trp	$y=1.21\times10^4x-5.27\times10^4$	0.9977	0.005–1.00	1.645	5.486
Fru-Pro	$y=3.37\times10^3x+4.88\times10^3$	0.9999	0.050–10.0	1.595	5.318
Fru-Phe	$y=8.87\times10^3x+4.82\times10^3$	0.9999	0.005 –1.00	1.354	4.516
Fru-Val	$y=1.14\times10^4x+5.32\times10^3$	0.9998	0.005 –1.00	1.763	5.877
Fru-Lle	$y=9.18\times10^3x-543$	0.9997	0.005 –1.00	2.113	7.045
Fru-Ala	$y=6.08\times10^3x+1.79\times10^4$	0.9998	0.005 –1.00	1.523	5.078
Fru-Glu	$y=5.61\times10^3x-1.31\times10^3$	0.9996	0.005 –1.00	1.827	6.092
Glucosamine	$y=5.97\times10^3x+1.85\times10^4$	0.9994	0.005 –1.00	1.922	6.408

1) Determination of 10 Amadori Compounds by LC-MS/MS

Validation of the method

- ✓ Inter-day precisions：1.0%~6.8%
- ✓ Intraday-precisions：4.0%~10.3%
- ✓ LOQ： 4.5~8.4 ng/mL
- ✓ Recovery rates： 85%~119%（ Burley tobacco ）
  93%~118%（ flue-cured tobacco ）
  84%~111%（ oriental tobacco ）
- Matrix effect: 88.9%-107%（Burley tobacco）
  89.0%-106%（flue-cured tobacco）
  87.4%~113%（oriental tobacco）

$$ME=\frac{\text{slope of calibration curve in matrix}}{\text{slope of calibration curve in solvent}}$$

2) Determination of 12 Amadori Compounds by LC-Q-TOFMS in combination with LC-MS/MS

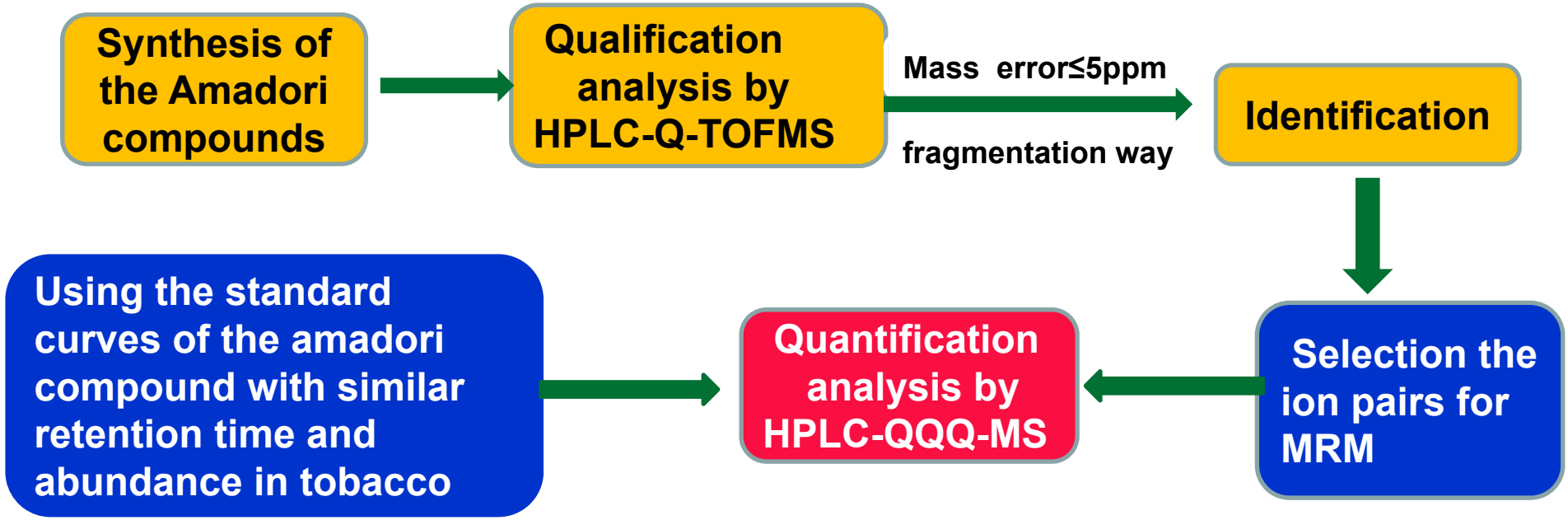
There are mainly 21 Amino acids with a certain amounts in tobacco, together with ammonium

There should be 22 glucose derived amadori compounds existed in tobacco in total theoretically.

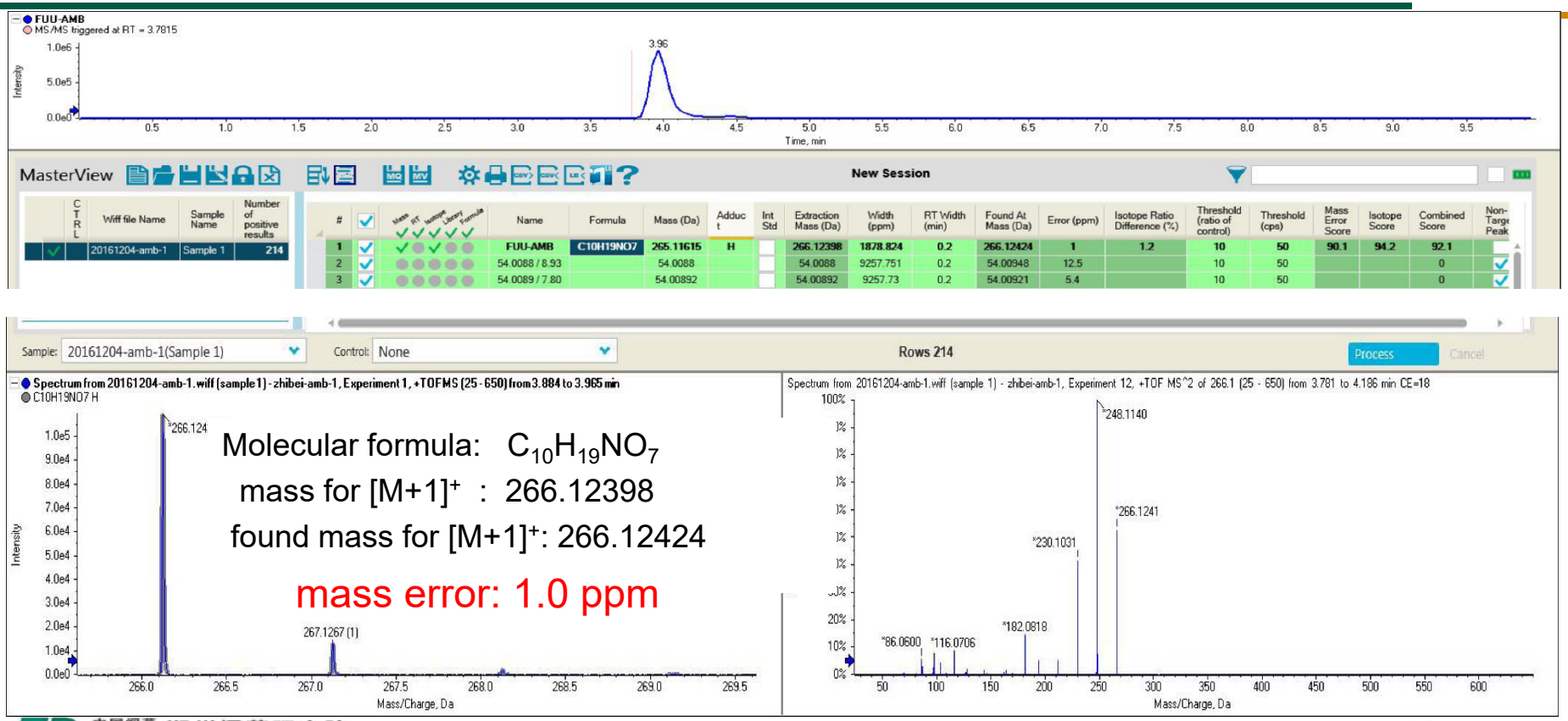
10 standards available

How to detect the other 12 amadori compounds?

2) Determination of 12 Amadori Compounds by LC-Q-TOFMS in combination with LC-MS/MS



Identification of Fructose-aminobutyric acid(Fru-Amb)

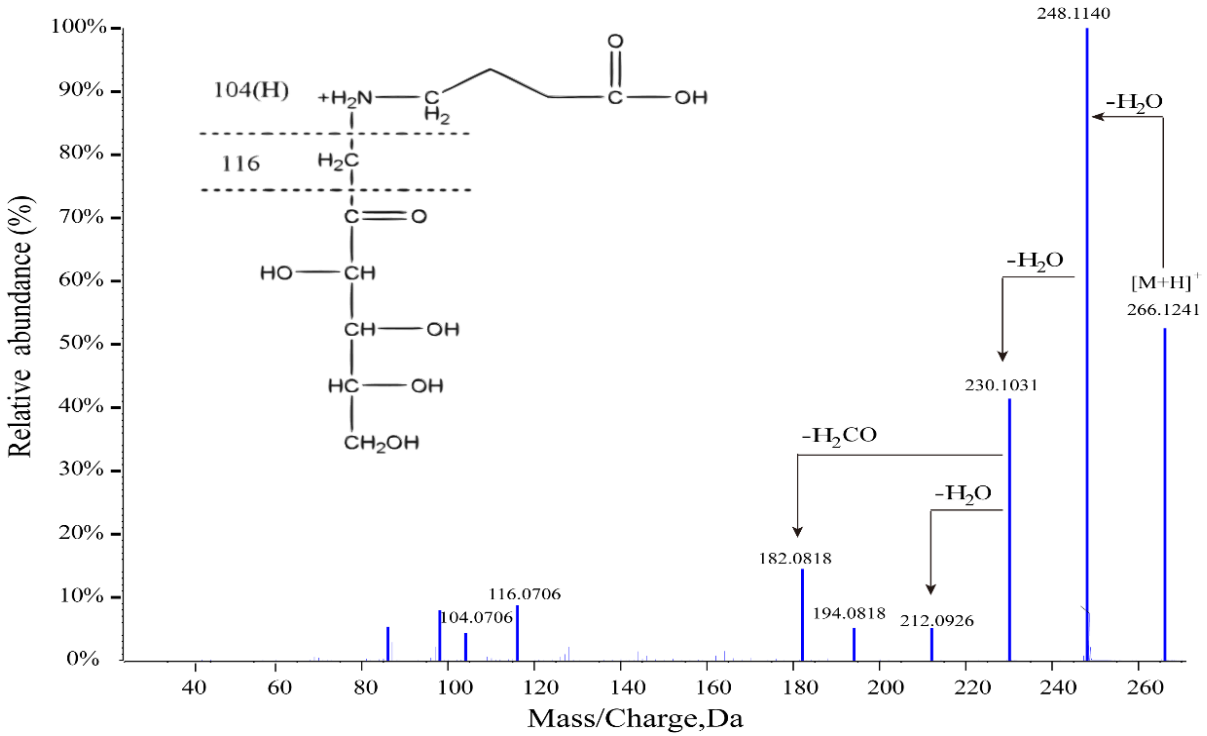


Molecular formula: $C_{10}H_{19}NO_7$

mass for $[M+1]^+$: 266.12398

found mass for $[M+1]^+$: 266.12424

mass error: 1.0 ppm

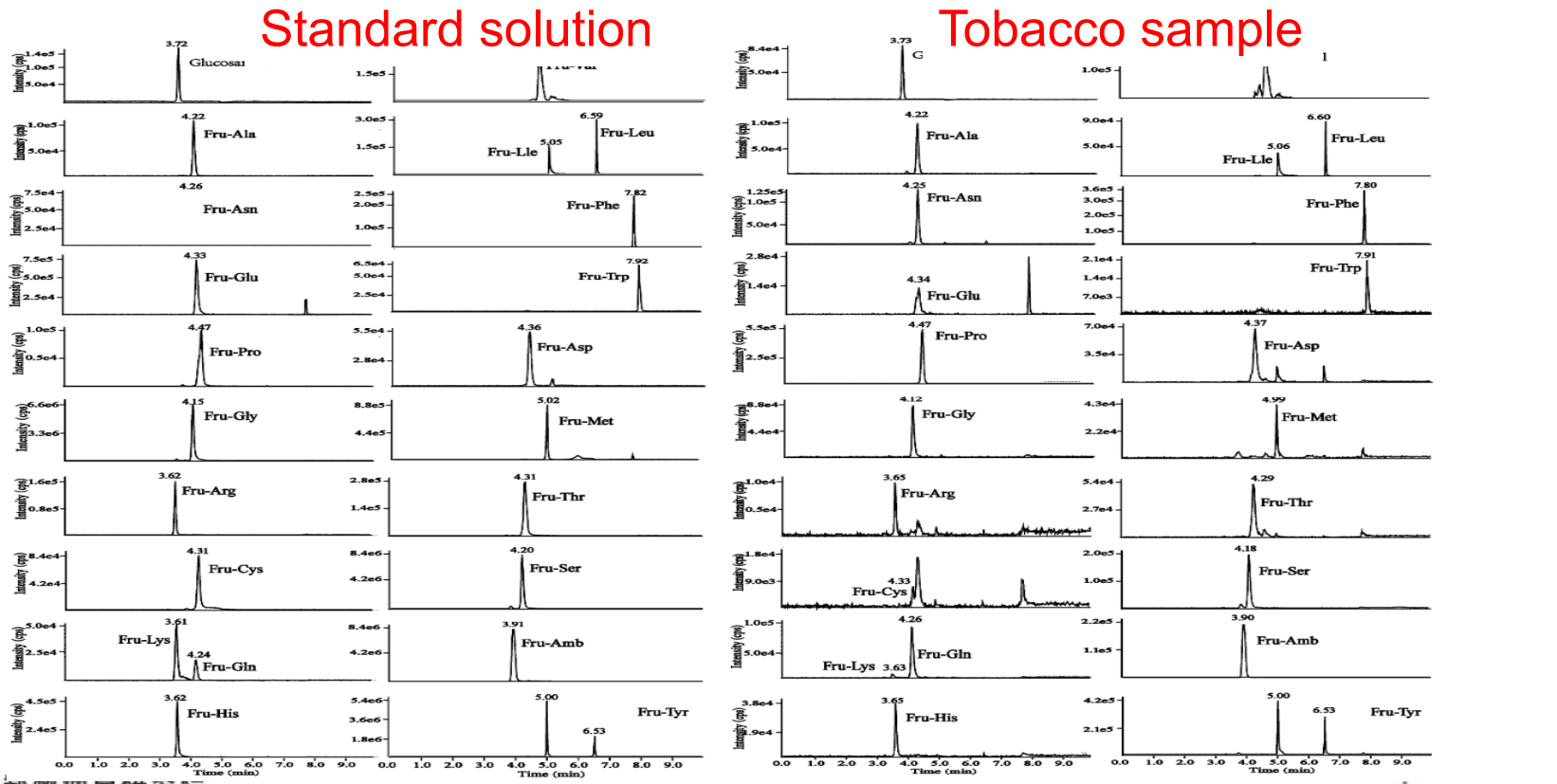


Ion pairs for MRM:
248.1/230.1
248.1/212.2

The fragmentation mechanism for Fru-Amb

Compound	Formula	Ion	Extracted mass(Da)	Found mass (Da)	Mass error (ppm)	Match rate
Fru-Amb	$C_{10}H_{19}NO_7$	$[M+H]^+$	266.12398	266.12424	1.0	92.1
Fru-Arg	$C_{12}H_{24}N_4O_7$	$[M+H]^+$	337.17232	337.17252	0.6	95.9
Fru-Asp	$C_{10}H_{17}NO_9$	$[M+H]^+$	296.09816	296.09805	-0.4	97.4
Fru-Cys	$C_9H_{17}NO_7S$	$[M+H]^+$	284.08040	284.07980	-2.1	82.4
Fru-Gln	$C_{11}H_{19}N_2O_8$	$[M+H]^+$	309.12979	309.12983	0.1	98.3
Fru-Gly	$C_8H_{15}NO_7$	$[M+H]^+$	238.09268	238.09206	-2.6	83.0
Fru-His	$C_{12}H_{19}N_3O_7$	$[M+H]^+$	318.13013	318.12942	-2.2	87.8
Fru-Lys	$C_{12}H_{24}N_2O_7$	$[M+H]^+$	309.16618	309.16687	2.2	85.6
Fru-Met	$C_{11}H_{21}NO_7S$	$[M+H]^+$	312.11170	312.11189	0.6	80.2
Fru-Ser	$C_9H_{17}NO_8$	$[M+H]^+$	268.10324	268.10357	1.2	91.3
Fru-Thr	$C_{10}H_{19}NO_8$	$[M+H]^+$	282.11889	282.11868	-0.7	91.0
Fru-Tyr	$C_{15}H_{21}NO_8$	$[M+H]^+$	344.13454	344.13419	-1.0	92.5

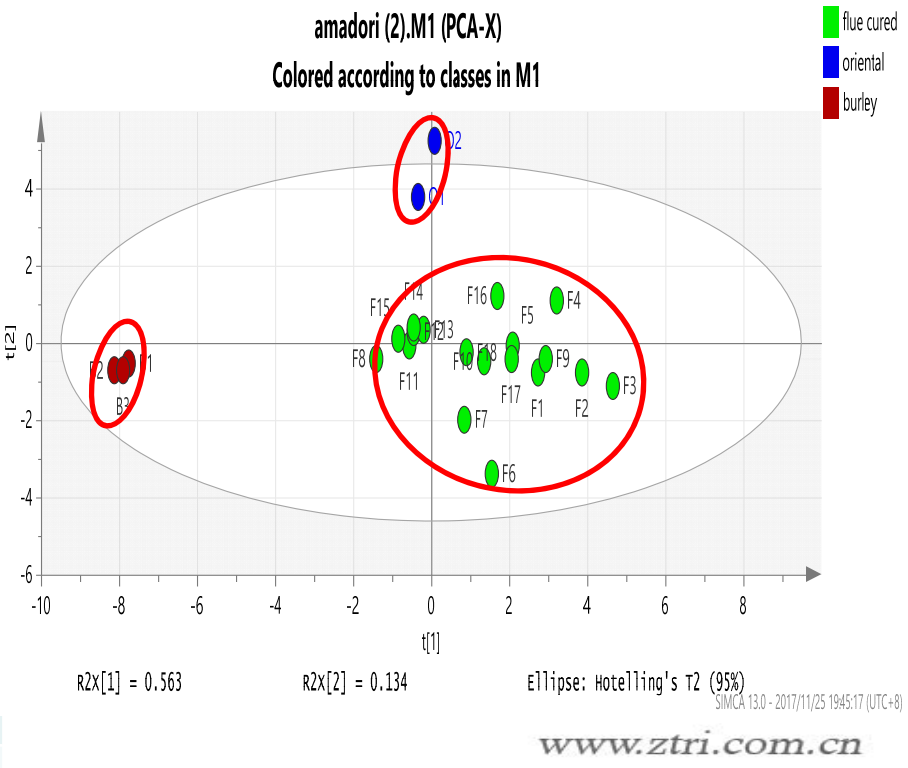
MRM chromatogram of the 22 Amadori compounds in the standard solution and a tobacco sample using HPLC-QqQ-MS/MS



The inter-day precision and intra-day precision during analysis of 120 Tobacco samples (n=2)

compound	Inter-day precision (n=6)	Intra-day precision (n=6)
Fru-Ala	6.8%	6.0%
Fru-Amb	8.4%	9.6%
Fru-Arg	6.4%	10.2%
Fru-Asn	6.8%	5.3%
Fru-Asp	6.2%	7.6%
Fru-Cys	5.7%	14.5%
Fru-Glu	7.9%	13.0%
Fru-Gln	13.8%	14.5%
Fru-Gly	4.4%	7.00%
Fru-His	2.6%	7.8%
Fru-Ile	5.4%	5.7%
Fru-Leu	9.8%	10.3%
Fru-Lys	11.1%	14.8%
Fru-Met	9.2%	9.3%
Fru-Phe	6.1%	9.4%
Fru-Pro	5.2%	4.2%
Fru-Ser	6.2%	6.4%
Fru-Thr	5.5%	6.8%
Fru-Trp	3.6%	4.0%
Fru-Tyr	3.7%	8.0%
Fru-Val	3.7%	4.6%
Glucosamine	6.2%	5.4%

compound	Burley	Oriental	Flue –cured
	Cotent (μg/g)		
Fru-Ala	67	1928	2898
Fru-Amb	94	1602	1971
Fru-Arg	4	12	17
Fru-Asn	230	2760	4386
Fru-Asp	187	933	1139
Fru-Cys	4	49	74
Fru-Glu	62	332	652
Fru-Gln	182	2589	1658
Fru-Gly	17	124	83
Fru-His	3	39	154
Fru-Ile	7	95	50
Fru-Leu	12	161	74
Fru-Lys	5	18	14
Fru-Met	3	38	42
Fru-Phe	36	516	537
Fru-Pro	126	3176	8434
Fru-Ser	3	54	68
Fru-Thr	13	84	68
Fru-Trp	14	44	494
Fru-Tyr	19	253	346
Fru-Val	24	429	536
Glucosamine	248	1552	530
Total amount	1358	16986	24226



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Conclusion

- By using HPLC-Q-TOF-MS, the 12 Amadori compounds were identified in the model reaction systems based on being matched with accurate mass and confirmed with the fragmentation way. And the ion pairs in MRM mode were selected.

- Using HPLC-QQQ-MS/MS, we quantified the 10 Amadori compounds with standards using external standard quantitative method, and semi-quantified the 12 Amadori compounds without standards using the standard curves of others with similar retention time and abundance in tobacco.

- Through large sample test, except 4 compounds which were unstable or of abundance close to LOQ, the intra-day precisions of 18 Amadori compounds were lower than 10%.

- Amadori compounds in flue-cured tobacco leaves and oriental tobacco leaves account for more than 1%. And these compounds contribute to the flavor of tobacco. They should be considered in monitoring the quality of tobacco leaves.

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Thank you !