Separation and Identification of Chlorogenic Acid and Related Impurities by High Performance Liquid Chromatography-Tandem Mass Spectrometry

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Abstract A method using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was established for analysis and identification of chlorogenic acid along with its related impurities. A Gemini C18 HPLC column was used with acetonitrile-water containing 0.1% formic acid as mobile phase. Eight related impurities of chlorogenic acid were identified and their structures were determined by using online HPLC-MS/MS and photodiode array detector. The method is quick and can be used directly to identify the structure of unknown trace substances in the sample of chlorogenic acid.

Key words high performance liquid chromatography-mass spectrometry/chlorogenic acid/related impurities/identification

1 Experimental
1.1 Instrumentation and Reagents

Hewlett-Packard HPLC system
芬尼根 LCQ DECA.

咖啡酸 [Sigma Chemical] 

绿原酸对照品 [中国药品生物制品检定所]、咖啡酸 [Sigma Chemical]、喹宁酸 [Sigma Chemical]、乙腈购于 [美国]。其他化学试剂皆为分析纯；实验用水为超纯水，临用前经 0.45 μm 微孔滤膜滤过。单体绿原酸样品 (含量为 99.85%) 由 [四川九章生物化工科技发展有限公司] 提供。

溶液制备

供试液：称取绿原酸样品适量，用流动相溶解，配成溶液，经 0.45 μm 微孔滤膜滤过，即得。

酸性氧化供试液：取绿原酸溶液，加过氧化氢溶液、盐酸溶液于水浴中加热 5 h，经 0.45 μm 微孔滤膜滤过，即得。

碱性氧化供试液：取绿原酸溶液，加过氧化氢溶液、氢氧化钠溶液于水浴中加热 5 h，经 0.45 μm 微孔滤膜滤过，即得。

色谱条件

色谱柱：Gemini C18 (5 μm)。流动相：乙腈-水 (含甲酸) (体积比为 7:3)。流速：1.0 mL/min。柱温：40 ℃。DAD 检测波长：190 ~ 800 nm。

质谱条件

电喷雾离子化源，喷口电压 4500 V。负离子检测模式；鞘气流量 496 L/min，辅助气流速：40 L/min，毛细管温度：150 ℃，毛细管电压：2200 V。

结果与讨论

取上述各供试液进样系统 (该系统并联有 DAD 检测器)，按照上述分析条件分离测定，分别采集分离组分的质谱图和紫外光谱图，记录相关数据。经测定，绿原酸及其相关杂质的图见图 1。根据鉴定结果对各相关杂质按相对保留时间排序，结果见表 1。

色谱组分的鉴定

经各组分检出的紫外光谱初步分析，2 号、3 号、7 号、8 号峰相应的紫外光谱均与绿原酸的紫外光谱一致，具有明显的光谱特征，表明这些物质具有类似的共轭结构。除 2 号色谱峰外，各色谱峰的紫外光谱中均存在 201,364,255 nm 的特征峰。因此，它们的主体结构应该与咖啡酸类似，主要的差异在于取代基不同。各色谱峰的负离子全扫描一级质谱中的主要离子为 [M - H]-，根据紫外图谱和 [M - H]-的差异、色谱保留时间以及二级质谱的特征离子等信息，参考文献进行化学成分的鉴别。

图 1 HPLC-DAD 色谱图

a. sample of chlorogenic acid; b. oxidized sample in acidic solution; c. oxidized sample in alkaline solution.

For peak identification see Table 1.

<table>
<thead>
<tr>
<th>Table 1 Related impurities in chlorogenic acid sample</th>
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<tbody>
<tr>
<td>Peak No. in Fig. 1</td>
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t_R relative retention time to chlorogenic acid.
Fig. 2  Fragmentation pathways of chlorogenic acid

1. $t_{u'} = 0.13$, M $-$ H$^-$, m/z 191
2. $t_{u'} = 0.18$, UV, 234 nm, M $-$ H$^-$, m/z 192
3. M, 346 nm, UV; m/z 280, 324 nm, M $-$ H$^-$, m/z 191
4. $t_{u'} = 0.39$, MS; 387 m/z, M $-$ H$^-$, m/z 239, 353, 247, 191, 173, 151, 137

Fig. 3  MS spectrum of quinic acid

Fig. 4  Fragmentation pathways of the 7|8-hydroxide of chlorogenic acid
第4期 田晨煦,等:高效液相色谱串联质谱法分离鉴定绿原酸及其相关杂质

号峰(的离子为,离子的谱中丰度较大的离子为,和。推断该组分为奎尼酸

号位与位为对称位置,因此位与位异构体的谱应一致。推断该组分为奎尼酸

位结合咖啡酰,应为绿原酸的同分异构体新绿原酸,结构式见图

新绿原酸的结构式

号峰(的离子为,和,与咖啡酸的谱略有差异,谱的主要碎片有,推断为咖啡酸的还原产物咖啡醛。

咖啡醛的裂解图

号峰(的谱与咖啡酸相同,相对分子质量比咖啡酸小。最大吸收波长为和,处有肩峰,与咖啡酸类似。推测其为咖啡酸的脱羧产物对乙烯基邻苯二酚。

对乙烯基邻苯二酚的裂解过程

号峰(的一级和二级质谱均与咖啡酸相同,最大吸收波长为和,处有肩峰,且与咖啡酸对照品的保留时间一致,故可确定其为咖啡酸。其裂解过程见图。

号峰(的一级和二级质谱均与绿原酸一致,推测其为绿原酸的同分异构体,谱与绿原酸有差异,最大吸收波长为和。结合号峰的解析,推断为奎尼酸位结合咖啡酰的异构体隐绿原酸,结构式见图。

图7 Fragmentation pathways of p-vinyl benzoatechgin

图8 Fragmentation pathways of caffeic acid

图9 Structure of cryptochlorogenic acid

讨论 由于单体绿原酸样品中相关杂质的含量较低,实验通过光照、氧化、高温、强酸、强碱等手段使其产生更多的杂质,色谱分离结果表明绿原酸主成分均能与降解产物完全分离,降解产物大致相同,不同反应条件所得杂质以某种降解产物为主,因此,选取降解产物较多的酸碱氧化的绿原酸溶液为代表进行鉴别。
Fig. 10 Fragmentation pathways of 3-cumaroylquinic acid

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M = 338
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M = 173
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m/z 157
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M + Na^- + 2H^- 719
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M + Na^- + 2H^- 739
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m/z 371 M + Na^- + 2H^- 739
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m/z 393 M + Na^- + 2H^- 739
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m/z 201 M + Na^- + 2H^- 719
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m/z 375 M + Na^- + 2H^- 739
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M + Na^- 2H^- 719
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M + Na^- 2H^- 739
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