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高效液相色谱-电感耦合等离子体质谱法分析研究西兰花中硒形态

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摘要: 硒是一种典型的"双功能"元素,摄入不足或摄入过量均会对人体健康产生不利影响,硒的生物活性不仅取决于硒含量,还与硒的化学形态密切相关,因此对食品中不同硒形态进行分析研究具有重要的意义。本文采用高效液相色谱-电感耦合等离子体质谱(HPLC-ICP/MS)联用技术分析研究了市售西兰花中硒酸根[Se(Ⅵ)]、亚硒酸根[Se(Ⅳ)]、硒代胱氨酸($SeCys_2$)、甲基硒代半胱氨酸(MeSeCys)、硒代蛋氨酸(SeMet)。以蛋白酶 XIV 和 Tris-HCl 缓冲溶液超声提取西兰花中硒形态,采用 C18 反相色谱柱为分析柱,10mmol/L 柠檬酸和 5mmol/L 己烷磺酸钠(pH=4.0,含 1%甲醇)为流动相,等度洗脱,8min 内可实现硒形态的有效分离测定,方法线性范围为 0.3~100.0μg/L,线性相关系数(r)均大于 0.999,Se(Ⅵ)、Se(Ⅳ)、MeSeCys、SeMet 的检出限在 1.2~6.0μg/kg(以 Se 计)范围内。对西兰花样品进行低、中、高三个浓度水平的加标回收试验,加标回收率为 81.9%~105.3%,相对标准偏差(RSD)均小于 5%。采用本方法分析欧盟有证标准物质——小麦粉(ERM® BC210a)中 SeMet 的测定值在其标准值范围内。实验结果表明建立的硒形态分析方法适用于西兰花中 Se(Ⅵ)、Se(Ⅳ)、MeSeCys、SeMet 的测定。检出的 11 个不同地区市售西兰花样品中硒形态主要为 MeSeCys,含量在 0.004~0.043mg/kg(以 Se 计)之间。对方法研究过程中发现的 $SeCys_2$ 稳定性差和不同类型西兰花中 Se(Ⅳ)加标回收率差异较大的问题进行分析探讨,通过改变蛋白酶 XIV 的用量考察了 $SeCys_2$ 的稳定性,结合对西兰花样品基质的分析研究,发现 $SeCys_2$ 稳定性与蛋白酶 XIV 含量和西兰花基质有关;根据对 3 种不同类型的西兰花样品中 Se(Ⅳ)加标回收试验结果及相关文献报道,推测样品中存在的大量酚类物质会影响 Se(Ⅳ)的分析测定。

关键词: 西兰花; 硒形态; 高效液相色谱-电感耦合等离子体质谱法; 蛋白酶 XIV

要点:

(1) 采用蛋白酶 XIV 和 Tris-HCl 缓冲溶液超声提取西兰花中硒形态。

(2) 采用 C18 反相色谱柱为分析柱,柠檬酸和己烷磺酸钠为流动相,HPLC-ICP/MS 分析西兰花中硒形态。

(3) 西兰花样品中硒形态主要为甲基硒代半胱氨酸(MeSeCys)。

(4) $SeCys_2$ 稳定性与蛋白酶 XIV 含量和西兰花基质有关。

中图分类号: P618.76; O657.63 **文献标识码:** A

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硒是人体必需的微量元素,具有多种形态,主要分为无机硒和有机硒,无机硒主要包括硒酸盐[Se(Ⅵ)]、亚硒酸盐[Se(Ⅳ)]等,有机硒主要包括硒代胱氨酸($SeCys_2$)、硒代蛋氨酸(SeMet)、甲基硒代半胱氨酸(MeSeCys)等硒代氨基酸,研究表明有机硒具有较高的生物活性和生物利用度[1-5],对健康有益。因此,在硒的营养效应受到日益关注的同时,硒和硒形态的分析研究越来越受到重视。由于硒形态分析与样品基质密切相关,硒形态自身不稳定以及提取试剂的影响[6],目前尚无统一的食品中硒形态分析检测标准。西兰花复含蛋白质、黄酮、多酚及维生素等[7-8]营养物质,且含硫代葡萄糖苷和较强的聚硒能力,具有抗氧化、抗癌等医用价值[9-10],对西兰花中硒形态分析研究具有重要的意义。

目前的硒形态分析方法主要有高效液相色谱-原子荧光光谱法(HPLC-AFS)[11-14]、高效液相色谱-电感耦合等离子体质谱法(HPLC-ICP/MS)[15-16]、液相色谱-高分辨率质谱如液相色谱-四极杆/静电场轨道阱高分辨质谱(LC-Q Exactive Orbitrap MS)[4]、气相色谱-串联质谱法(GC-MS/MS)[17]、毛细管电泳-电感耦合等离子体质谱法(CE-ICP/MS)[18]。液相色谱-高分辨率质谱根据化合物的分子离子、碎片离子信息及分子裂解机理等确定分子结构,进行定性定量分析,可对无标准物质的硒形态进行鉴定分析,但因价格昂贵其应用受到较大的限制;GC-MS/MS 适用于易挥发硒形态的测定,对于难挥发性硒形态需经过衍生化处理,而多数样品需要衍生,分析步骤繁琐且易发生形态转化;CE-ICP/MS 虽然分离度好,但受接口、进样以及化学基体效应等因素的限制,应用受限[19];HPLC-AFS 和 HPLC-ICP/MS 因接口简单、灵敏度高、方便快捷等优点已成为硒形态检测的主流方法。HPLC-AFS 以操作简单、成本低[20]广泛应用于硒形态分析,但 HPLC-AFS 灵敏度相对较低,对于硒含量低的样品存在一定的局限性;ICP-MS 具有高选择性和高灵敏度,与 HPLC 联用是硒形态最有力的分析技术。目前对于西兰花中硒形态的分析研究多采用 HPLC-AFS。陆晓奇等[12]将富硒植物干粉溶于 Tris-HCl 缓冲液并依次加入纤维素酶、蛋白酶 K 和蛋白酶 XIV,于气浴恒温振荡器中酶解,采用 HPLC-UV-AFS 检测,样品提取效果好,但提取时间近 42.5h;刘为等[13]采用蛋白酶 K 和蛋白酶 E 对富硒农产品进行酶解,水浴振荡提取,采用 HPLC-HG-AFS 分析了富硒西兰花干粉中 4 种硒形态 $SeCys_2$、SeMet、MeSeCys、硒代乙硫氨酸(SeEt),分析效果较好,但方法检出限为 0.86~2.79μg/L,灵敏度有待进一步提高。Pedrero 等[21]采用 HPLC-ICP/MS 对富硒西兰花中 Se(Ⅳ)、$SeCys_2$、SeMet、MeSeCys 进行检测,10min 内实现 4 种硒形态的分离,分析时间较长。

为了提高检测灵敏度,缩短分析时间,本研究应用 HPLC-ICP/MS,以 ZORBAX SB-Aq C18 反相离子对色谱柱为分析柱,对市售西兰花中 Se(Ⅵ)、Se(Ⅳ)、$SeCys_2$、MeSeCys、SeMet 共 5 种硒形态进行研究,为研究西兰花的营养价值提供支持。

1　实验部分

1.1　仪器

1260 型高效液相色谱仪及 7700x 型电感耦合等离子体质谱仪(美国 Agilent 公司);Milliplus 2150 超纯水处理系统(美国 Millipore 公司);超声波清洗机(宁波新芝生物科技股份有限公司);冷冻离心机(美国 Beckman 公司)。

1.2　样品与主要试剂

供试样品:①市售西兰花:从北京市的超市及网上采购的山东、广东、河北、云南等各地西兰花样品;②选一购于浙江临海的市售西兰花样品均质(为 a)并进行冷冻干燥成粉末(为 b);③经含 Se(Ⅳ)的硒肥强化后的西兰花冷冻干燥粉末(为 c,北京农林科学院农产品加工与食品营养研究所提供)。

超纯水(电阻率 18.2MΩ·cm,由超纯水处理系统制备);柠檬酸(优级纯,国药集团化学试剂有限公司);己烷磺酸钠(优级纯,国药集团化学试剂有限公司);甲醇(HPLC 级,美国 Sigma 公司);氨水(优级纯,国药集团化学试剂有限公司);蛋白酶 XIV(美国 Sigma 公司);三羟基甲基氨基甲烷盐酸盐(99.0%,美国 Sigma 公司);亚硒酸根离子溶液(GBW10032)、硒酸根离子溶液(GBW10033)、甲基硒代半胱氨酸(GBW10088)、硒代蛋氨酸(GBW10034)、硒代胱氨酸(GBW10087)购于中国计量科学研究院,欧盟有证标准物质富硒小麦粉(ERM®-BC210a,购于 LGC 标准品公司)。

本文中的硒含量均是以 Se 计。

1.3　仪器条件

1.3.1　色谱条件

色谱柱:安捷伦 ZORBAX SB-Aq C18(250mm×4.6mm, 5μm);流动相:10mmol/L 柠檬酸及 5mmol/L 己烷磺酸钠(含 1%甲醇,pH=4.0);流速

0.8mL/min;进样量 20μL。

1.3.2 质谱条件

RF 入射功率 1550W;载气:高纯氩气;载气流速 0.65L/min;补偿气流速 0.45L/min;冷却气流速 15L/min;采样锥、截取锥:镍锥;射频电压 1.80V;采样深度 8.0mm;泵速 0.3r/s;检测同位素:^{78}Se。

1.4 硒形态的样品前处理

取 1.0~1.5g 西兰花样品于聚丙烯离心管中,若冷冻干燥的粉末则取 0.3g,加入 12mL 100mmol/L 三羟基甲基氨基甲烷盐酸盐缓冲液(Tris-HCl,pH=7.4,含 6mg/mL 的蛋白酶 XIV),涡旋混匀后于 37℃下加热超声 3h,在 4℃下 9000r/min 离心 10min,取上清液经 0.22μm 水系滤膜过滤,同时做试剂空白。

2 结果与讨论

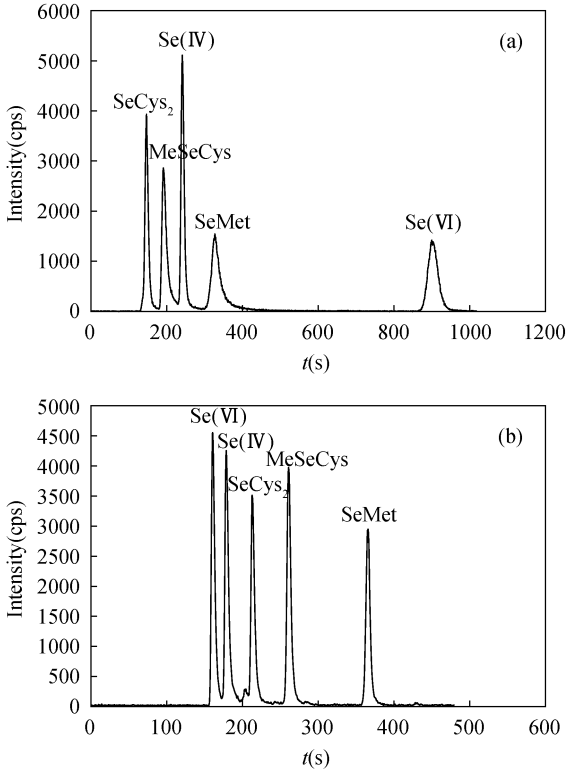
2.1 色谱条件的选择

硒形态分析常用 Hamilton PRP-X100 阴离子交换色谱柱(4.1mm×250mm,10μm)[22-25]和反相离子对色谱柱[26-27],其中以 Hamilton PRP-X100 色谱柱居多,但在分析植物样品时,由于植物样品以酶解法提取时会有较多的酶残留在提取液中,这些大分子物质会对色谱柱造成一定的损害,导致色谱柱的使用寿命缩短[28]。Hamilton PRP-X100 与磷酸盐体系分析 $SeCys_2$、MeSeCys、Se(Ⅳ)、SeMet 和 Se(Ⅵ)这 5 种硒形态在 16min 内可完成分离,SeMet 和 Se(Ⅵ)灵敏度明显低于其他三个硒形态,色谱图见图 1a。

张珂等[26]和姚晓慧等[27]使用 ZORBAX SB-Aq C18 反相离子对色谱柱与柠檬酸体系加入离子对试剂——已烷磺酸钠可以在 8min 内实现所测硒形态的有效分离,且各硒形态灵敏度较高。其中姚晓慧等[27]采用等度洗脱方式更简便,时间更短。因此本实验采用此色谱条件进行分析研究,色谱图见图 1b。

2.2 样品前处理条件优化

植物中硒的提取方法有超纯水提、酸提[29]、醇提以及酶解法[23]。采用超纯水、酸、醇提取一般只能提取无机硒及水溶性氨基酸,对植物体内与大分子蛋白结合的硒提取效果较差,且酸提取会破坏植物体中硒形态。酶解法可将与蛋白结合的硒分离且方法温和,有利于有机硒的提取[28],该法时间长,为缩短提取时间和减少硒形态转变,可采用超声辅助萃取和微波辅助萃取。本研究采用超声辅助提取,对提取试剂和蛋白酶的种类、用量以及提取时间进行优化。



(a) Hamilton PRP-X100 色谱柱; (b) ZORBAX SB-Aq C18 色谱柱。

图 1 五种形态硒混合标液在不同色谱柱下的色谱图(10μg/L)

Fig. 1 Chromatograms of 5 forms of selenium mixed standard solution under different columns (10μg/L).
(a) Hamilton PRP-X100 column; (b) ZORBAX SB-Aq C18 column.

2.2.1 提取试剂的选择

本研究选择一个硒含量为 0.81mg/kg 的西兰花样品,以超纯水、缓冲溶液、蛋白酶 XIV 和复合蛋白酶作为提取剂,通过超声辅助提取考察对西兰花样品的提取效果。表 1 实验数据表明,蛋白酶 XIV 作为提取试剂时硒的提取效果最佳。因此本研究选用蛋白酶 XIV 为提取试剂进行后续硒形态的分析研究。

2.2.2 蛋白酶 XIV 用量的优化

选用蛋白酶 XIV 为提取试剂,为进一步考察蛋白酶 XIV 含量对提取效果的影响,应用含蛋白酶 XIV 浓度为(2、4、6、8mg/mL)的 Tris-HCl 缓冲液(pH=7.4)对西兰花中 5 种硒形态进行提取,实验结果见图 2。结果表明,5 种硒形态含量随蛋白酶 XIV 浓度增加而增加,当蛋白酶 XIV 浓度为 6mg/mL 时提取效果最好;当蛋白酶 XIV 浓度增加到 8mg/mL 时 $SeCys_2$、MeSeCys、SeMet 的提取效果有所下降,Se(Ⅳ)和 Se(Ⅵ)提取效果无差别。因此,选择蛋白酶 XIV 浓度为 6mg/mL。

表 1　不同提取剂对西兰花样品中硒形态提取效果的影响

Table 1　Extraction results of selenium speciation in broccoli sample using different extractants. As shown in the table, proteinase XIV is the best to use for extracting.

提取剂	Se(Ⅵ)含量 (mg/kg)	Se(Ⅳ)含量 (mg/kg)	$SeCys_2$ 含量 (mg/kg)	MeSeCys 含量 (mg/kg)	SeMet 含量 (mg/kg)	5 种硒形态含量之和 (mg/kg)
超纯水	0.029	0.020	0.019	0.136	0.051	0.255
100mmol/L Tris-HCl 缓冲液	0.025	0.021	0.017	0.124	0.012	0.199
蛋白酶XIV	0.026	0.018	0.042	0.140	0.300	0.526
复合蛋白酶	0.028	0.015	0.000	0.108	0.244	0.395

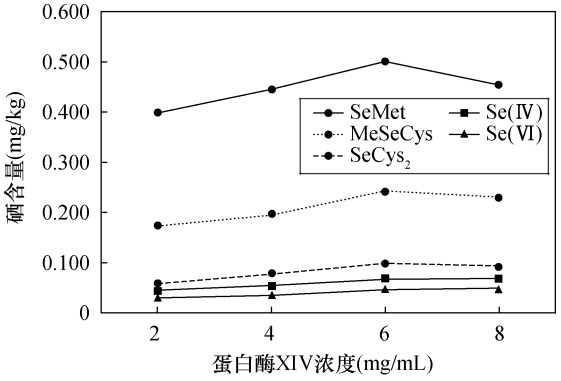


图 2　不同浓度的蛋白酶 XIV 对西兰花样品中 5 种硒形态的提取效果

Fig. 2　Effect of different concentrations of proteinase XIV on the extraction of five selenium speciation from broccoli samples. As can be seen from the graph, the content of the five selenium speciation increases and then decreases with the increase of concentration of proteinase XIV. The best extraction efficiency was reached when the concentration of proteinase XIV was 6mg/mL.

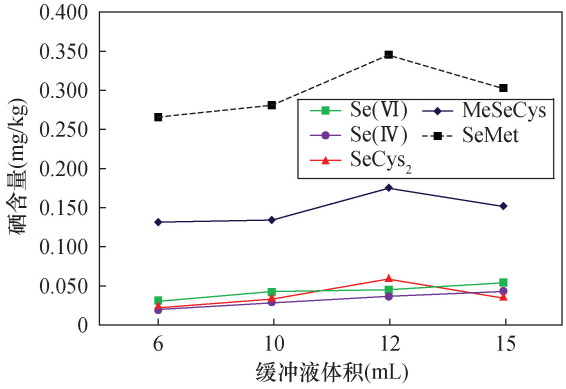


图 3　不同体积的缓冲溶液对西兰花样品中 5 种硒形态提取效果的影响

Fig. 3　Effects of different volumes of buffer solution on the extraction of five selenium speciation from broccoli samples. The graph shows that the best extraction results were obtained when the buffer solution was added at 12mL.

2.2.3　缓冲溶液加入量的优化

Tris-HCl 缓冲溶液作为核酸和蛋白质溶剂，在适宜的 pH 下，与蛋白酶 XIV 配合使用作为硒形态分析的提取剂具有较好的效果[30]。本研究选择 Tris-HCl 缓冲溶液为提取溶液，并考察了缓冲溶液（pH=7.4，含 6mg/mL 蛋白酶 XIV）加入量（6、10、12、15mL）对 5 种硒形态的提取效果，结果见图 3。

随缓冲溶液体积增加，各形态的提取效果先逐渐升高后降低，与林樾[31]研究结果一致，可能是适宜的缓冲溶液体积使样品溶解度变高，蛋白质分子更易扩散，酶对样品的水解更加完全，从而达到更好的提取效果。当到达最大溶解度后，随缓冲溶液体积提高，样品浓度变小导致提取率下降。另外提取溶剂的增加会导致硒浓度降低，因此最终 Tris-HCl 缓冲溶液的加入体积为 12mL。

2.2.4　提取时间的优化

为考察提取时间对提取效果的影响，设置了 1h、3h、5h、7h 四个提取时间对 5 种硒形态的提取效果进行研究。实验结果显示，提取时间从 1h 延长至 3h，提取出的各硒形态含量均有提高（仅 $SeCys_2$ 有轻微下降）。3h 与 5h 提取效果相当，略低于 7h 提取效果，增加提取时间提取效果未有显著变化，长时间酶解条件下会引起 SeMet 以及 $SeCys_2$ 稳定性降低[32-33]，因此最终选择提取时间为 3h，既缩短分析时间也减少硒形态间的转换。

2.3　方法线性范围和检出限

分别配制 0.0、0.5、1.0、5.0、10.0、25.0、50.0 和 100.0μg/L 的 Se(Ⅵ)、Se(Ⅳ)、$SeCys_2$、MeSeCys 和 SeMet 共 5 种硒形态混合标准系列，在优化好的实验条件下进行线性范围实验。实验结果表明在 0.3~100.0μg/L 范围内 5 种硒形态线性关系良好，线性相关系数（r）均大于 0.999。

在空白样品中分别加入 0.5、0.4、0.3、0.2、0.1、0.05μg/L 的标准溶液进行硒形态含量测定,根据各硒形态的测定结果信号值与 3 倍信噪比(S/N)相对应的浓度为硒形态的检出限,定量限为检出限的 3 倍。当市售西兰花样品的称样量为 1.0g,加入提取剂为 12mL 时,计算方法检出限,结果见表 2。

2.4　方法正确度和重复性

2.4.1　样品加标回收和精密度试验

选取一个市售西兰花样品,同时制备 6 个样品,分别添加三个不同浓度水平的 5 种硒形态混合标准溶液进行加标回收和精密度试验,测定结果见表 3。Se(Ⅵ)、Se(Ⅳ)、MeSeCys、SeMet 的加标回收率在 81.9%~105.3%范围内,$SeCys_2$ 的加标回收率为 7.91%~10.5%。5 种硒形态的 RSD 均小于 5%。

针对西兰花中 $SeCys_2$ 加标回收率低的现象进行了进一步研究,在空白试剂中加入不同浓度(1、2、4、6mg/mL)的蛋白酶 XIV 及 10μg/L 的 $SeCys_2$ 标准溶液,应用所建立的方法进行加标回收试验,考察提取体系对 $SeCys_2$ 稳定性的影响,发现随蛋白酶 XIV 浓度升高,$SeCys_2$ 信号值降低,出现的三个未知峰(U_1、U_2、U_3)信号值逐渐升高,说明蛋白酶 XIV 的浓度会影响 $SeCys_2$ 的稳定性,测定的色谱图见图 4。虽然经优化选择的含蛋白酶 XIV 提取体系对西兰花中硒形态的提取效果较好,但蛋白酶 XIV 浓度会影响 $SeCys_2$ 的稳定性,并依据西兰花样品中 $SeCys_2$ 加标回收率低的情况,说明蛋白酶 XIV 浓度和西兰花基质会影响 $SeCys_2$ 的准确测定。

表 2　方法线性方程、相关系数和检出限

Table 2　Linear equations, correlation coefficients, and detection limit of the method.

硒形态	线性范围 (μg/L)	线性方程	相关系数 (r)	定量限 (μg/kg)	方法检出限 (μg/kg)
Se(Ⅵ)	0.9~100.0	$y=2243.1x-650.8$	0.9999	10.8	3.6
Se(Ⅳ)	0.6~100.0	$y=2165.7x-412.7$	0.9999	7.2	2.4
$SeCys_2^*$	1.0~100.0	$y=2183.6x-765.0$	1.0000	–	–
MeSeCys	0.3~100.0	$y=2385.0x-1544.4$	0.9999	3.6	1.2
SeMet	1.5~100.0	$y=2169.5x-607.9$	1.0000	18.0	6.0

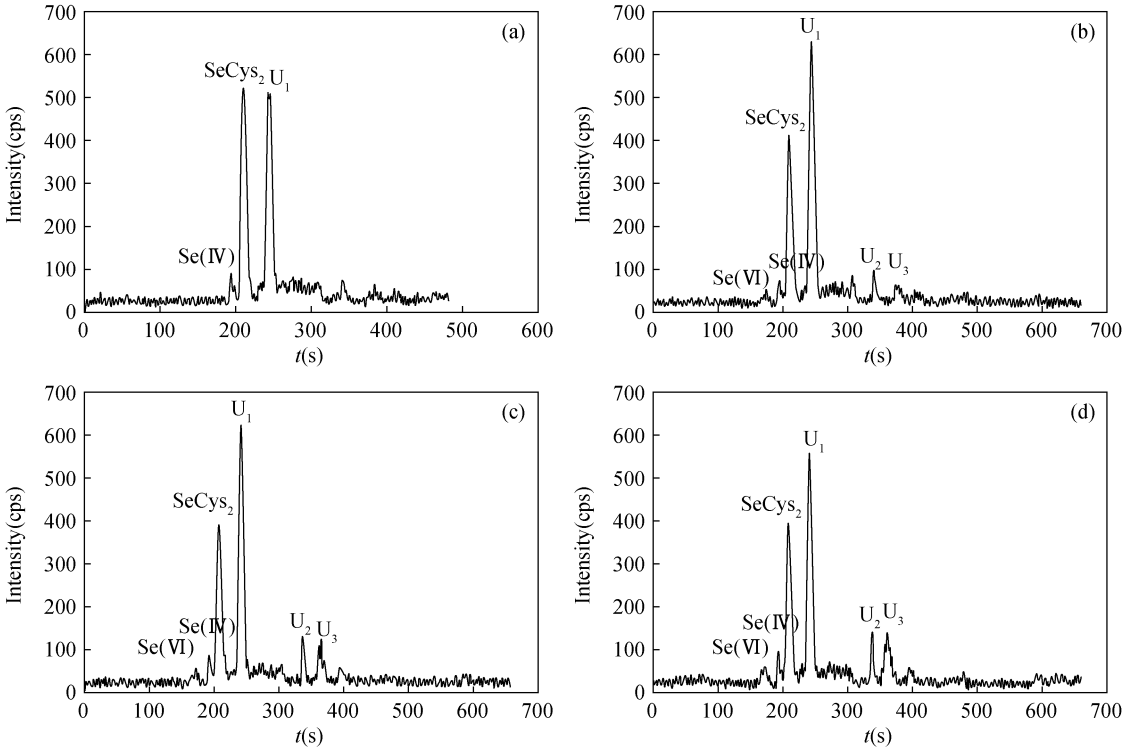
注:“*”表示因 $SeCys_2$ 的加标回收率低于 80%无法准确定量,故未计算方法检出限。

Note: “*” indicates that the detection limit of the method was not calculated because the spiked recovery of $SeCys_2$ was less than 80% and could not be accurately quantified.

表 3　西兰花精密度及加标回收率测定结果($n=6$)

Table 3　Determination results of precision and recovery rate of broccoli ($n=6$).

硒形态	本底值 (mg/kg)	加标量 (mg/kg)	6 次测定值 (mg/kg)						加标回收率 (%)	RSD (%)
Se(Ⅵ)	ND	0.12	0.123	0.126	0.123	0.125	0.126	0.124	102.2~105.3	1.0
		0.36	0.366	0.367	0.360	0.366	0.369	0.366	100.0~102.1	1.6
		0.60	0.609	0.620	0.594	0.608	0.614	0.604	99.0~103.3	1.3
Se(Ⅳ)	ND	0.12	0.099	0.100	0.099	0.100	0.100	0.100	82.7~85.2	1.0
		0.36	0.305	0.295	0.296	0.295	0.296	0.301	81.9~85.6	1.7
		0.60	0.501	0.505	0.502	0.500	0.505	0.497	82.8~84.1	0.7
$SeCys_2$	ND	0.12	0.009	0.010	0.010	0.010	0.011	0.010	7.91~8.77	4.4
		0.36	0.036	0.034	0.034	0.034	0.034	0.036	9.47~9.97	2.0
		0.60	0.060	0.061	0.058	0.063	0.063	0.062	9.74~10.5	2.5
MeSeCys	ND	0.12	0.107	0.106	0.107	0.108	0.108	0.106	88.1~89.7	0.7
		0.36	0.323	0.311	0.313	0.315	0.317	0.317	86.4~89.6	1.4
		0.60	0.544	0.542	0.544	0.554	0.553	0.554	90.4~92.4	1.3
SeMet	ND	0.12	0.126	0.124	0.125	0.125	0.127	0.127	98.4~102.9	0.7
		0.36	0.350	0.354	0.349	0.350	0.353	0.353	97.0~98.2	0.8
		0.60	0.591	0.595	0.595	0.599	0.617	0.605	98.4~102.9	1.9



(a) 蛋白酶 XIV 浓度为 1mg/mL; (b)蛋白酶 XIV 浓度为 2mg/mL; (c)蛋白酶 XIV 浓度为 4mg/mL; (d)蛋白酶 XIV 浓度为 6mg/mL。

图 4　不同浓度蛋白酶 XIV 对 $SeCys_2$ 标准溶液稳定性的影响

Fig. 4　Effect of different concentrations of proteinase XIV on stability of $SeCys_2$ standard solutions. The concentration of proteinase XIV is: (a) 1mg/mL; (b) 2mg/mL; (c) 4mg/mL; (d) 6mg/mL.

另外,对西兰花进行 Se(Ⅳ)加标回收试验时发现一个现象,即不同的西兰花样品对 Se(Ⅳ)的测定有影响。对选择的三个西兰花样品(a、b、c)进行 Se(Ⅳ)加标回收试验,称取一定的样品,加入 1.2mL 浓度为 100μg/L 的 Se(Ⅳ)标准溶液和提取试剂,按所建分析方法进行分析测定,发现三个样品的加标回收率依次为:81.6%>69.9%>1.5%,进行 Kruskal-Wallis 秩和检验,三者加标回收率具有显著性差异(P<0.05),具体结果见表 4。

西兰花粉末(c),即经硒强化后的西兰花冷冻干燥粉末 Se(Ⅳ)加标回收率明显降低,进一步增加蛋白酶 XIV 的用量到 8mg/mL,增加提取试剂体积至 12mL,提取效果并无明显改变,说明不存在因酶含量以及提取液体积不足引起竞争提取的问题。推测可能是样品本身存在的某些物质会影响 Se(Ⅳ)加标回收率。初步推测源于西兰花中存在的酚类化合物影响了 Se(Ⅳ)的测定,Cuderman 等[34]报道了类似的现象,曾将酚类化合物(单宁和芦丁)按照 1∶100(w/w)的比例加入到硒标准溶液中时,37℃采用蛋白酶酶解 24h 后发现 Se(Ⅳ)响应值下降 20%,说明酚类化合物会影响 Se(Ⅳ)测定。Tian 等[35]报道在西兰花的幼苗阶段施加硒酸钠发现酚酸(酚类化合物的一种)含量增加;同样,Gui 等[36]研究发现,在西兰花结球期,施加硒酵母和亚硒酸钠

表 4　三个不同的西兰花中 Se(Ⅳ)加标回收实验结果(n=3)

Table 4　Analytical results of spiked recovery test of Se(Ⅳ) for three broccoli samples (n=3).

样品名称	本底浓度 (mg/kg)	加标量 (mg/kg)	3 次测定加标回收率 (%)			平均加标回收率 (%)	H 值	P 值
西兰花(a)	ND	0.11	81.3	81.0	81.1	81.1		
西兰花粉末(b)	ND	0.40	68.1	68.4	72.1	69.5	7.20	0.027*
西兰花粉末(c)	0.008	0.40	1.55	1.53	1.53	1.53		

注: ND 表示低于检出限;"*":P 值小于 0.05 为差异具有统计学意义。

Note: ND indicates below detection limit; "*" indicates that p-value of less than 0.05 is considered a statistically significant difference.

发现酚酸含量明显上升,因此,推测 c 样品中 Se(Ⅳ)加标回收率显著下降是由于西兰花样品中酚类物质含量较高造成的,具体原因有待研究。

2.4.2　方法正确度验证

由于目前无西兰花基质的有证标准物质,于是选用欧盟有证标准物质小麦粉(ERM® BC210a,标准值为 11.03±1.05mg/kg,以 Se 计)进行试验,采用本研究建立的方法对 ERM® BC210a 中 SeMet 进行测定,SeMet 的测定值为 10.11±0.05mg/kg(n=3),测定值在其标准值范围内。

2.5　样品分析

应用所建立的分析方法对从山东、北京、广东、河北、云南等地采购的 20 多个市售西兰花样品进行硒形态分析,发现在 11 个检出硒形态的样品中主要形态为 MeSeCys,含量在 0.004~0.043mg/kg 之间,部分样品中还存在少量的 Se(Ⅵ)、Se(Ⅳ)和 SeMet,但含量均低于定量限,样品色谱图见图 5。样品色谱图显示,在保留时间为 340s 和 550s 左右,还存在两个未知的含硒化合物(U_a、U_b),具体物质有待进一步研究。已有文献[37-38]报道,西兰花中存在较高含量的 MeSeCys,源于西兰花作为十字花科中的次级聚硒植物,可通过甲基化的方式转变硒的储存方式[13]。

同时采用此方法对经含 Se(Ⅳ)的硒肥强化后的西兰花冷冻干粉进行分析。结果显示,经含 Se(Ⅳ)硒肥强化后的西兰花冷冻干粉中硒形态含量依次为 SeMet、MeSeCys 和少量的 $SeCys_2$,这与陆晓奇等[12]对富硒植物硒形态研究中得出富硒西兰花中硒主要存在形态为 MeSeCys(37.1%)、SeMet(27.8%)和 $SeCys_2$(25.9%)以及刘为等[13]实验结果显示富硒西兰花蛋白中 $SeCys_2$、SeMet 和 MeSeCys 占比高,结果近似。

市售西兰花中主要检出 MeSeCys,推测经硒肥强化后,硒在植物体中富集,转化为不同形态的有机硒。虽然本研究中 $SeCys_2$ 加标回收率不理想,但如果样品中存在 $SeCys_2$ 且含量较高时仍可以分析检出。

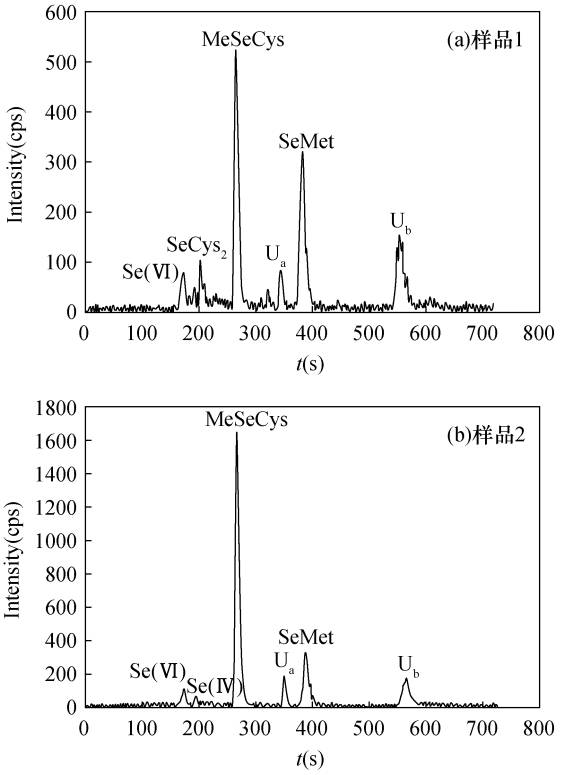


图 5　样品色谱图

Fig. 5　Chromatograms of samples. The chromatogram shows that the predominant speciation of selenium in the sample is methylselenocysteine.

3　结论

本研究通过对样品提取、分析条件的选择和优化,选择了含蛋白酶 XIV 的 Tris-HCl 缓冲溶液进行样品提取,建立了 HPLC-ICP/MS 测定市售西兰花中 Se(Ⅳ)、Se(Ⅵ)、MeSeCys、SeMet 的方法,对采集的全国不同地区市售的 20 多份西兰花样品进行分析测定,结果表明市售西兰花中硒形态以 MeSeCys 为主,存在少量 Se(Ⅵ)、Se(Ⅳ)和 SeMet,也存在少量未知含硒化合物。

在分析研究中发现,$SeCys_2$ 稳定性受蛋白酶 XIV 浓度及西兰花样品基质影响,同时推测若样品中存在大量酚类物质将会影响 Se(Ⅳ)的测定,具体原因有待进一步分析探究。

Selenium Speciation in Broccoli by High Performance Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometry

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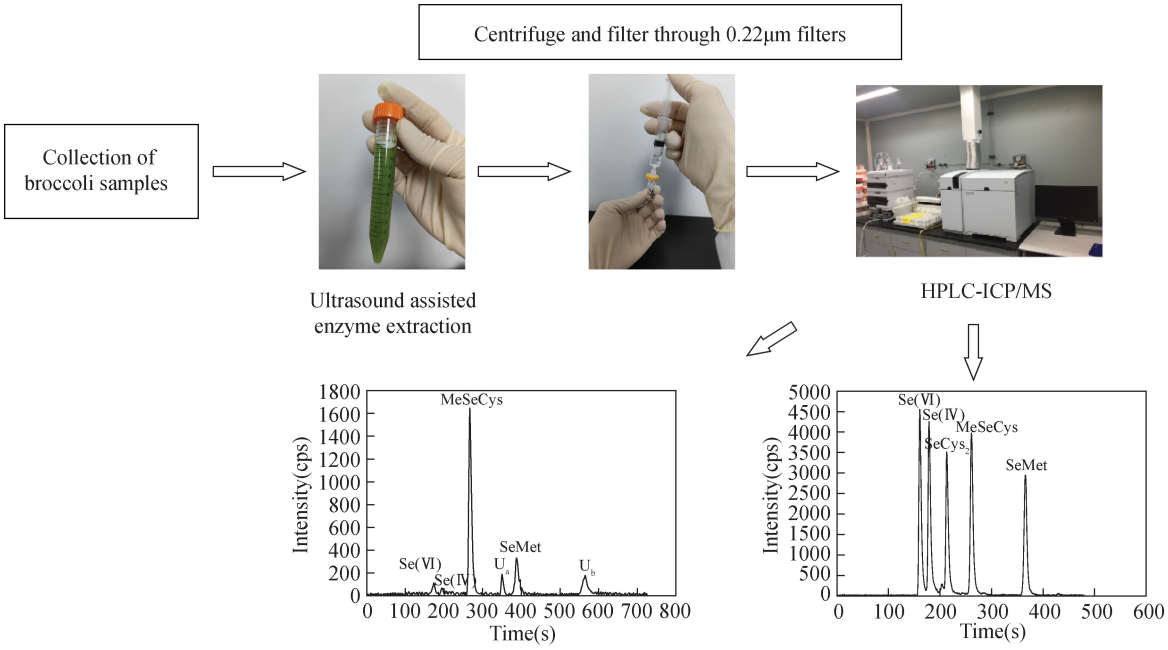
HIGHLIGHTS

(1) Selenium speciation in broccoli was extracted by proteinase XIV and Tris-HCl buffer solution.
(2) HPLC-ICP-MS equipped with ZORBAX SB-Aq C18 reversed-phase column with 10mmol/L citric acid and 5mmol/L sodium hexane-sulfonate as mobile phase was applied to analyze the selenium speciation in broccoli.
(3) Methylselenocysteine is the main selenium speciation in broccoli.
(4) The stability of the $SeCys_2$ standard solution is influenced by the proteinase XIV content and the sample matrix.



ABSTRACT

BACKGROUND: Selenium is an essential trace element and a typical bifunctional element that can affect human health if consumed in insufficient or excessive amounts. The biological activity of selenium depends not only on its intake level but also on its chemical speciation. Selenium comes in various speciation and is divided mainly into inorganic and organic selenium. Inorganic selenium includes selenate [Se(Ⅵ)] and selenite [Se(Ⅳ)], and organic selenium mainly includes selenocysteine ($SeCys_2$), selenomethionine (SeMet), and methylselenocysteine (MeSeCys). It has been found that organic selenium has high bioactivity and bioavailability. At present, while the nutritional effects of selenium are drawing more and more attention, it is very important to analyze and study the

different speciation of selenium in food. Since the analysis of selenium speciation is closely related to the sample matrix, the extraction efficiency and stability of different selenium speciation are also related to many factors. At present, the analysis method of selenium speciation in food is still in the research stage. Broccoli is rich in nutrients, such as protein, flavonoids, polyphenols, and vitamins, and is widely loved by people because it contains many kinds of thioglucosides and has a strong ability to gather selenium, which has antioxidant and anti-cancer medical values. Therefore, the analysis and study of selenium speciation in broccoli is of some significance.

OBJECTIVES: To establish a method for the determination of Se(Ⅵ), Se(Ⅳ), $SeCys_2$, MeSeCys, and SeMet in commercial broccoli by high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS).

METHODS: Firstly, the chromatographic conditions were selected by examining the separation and sensitivity of Se(Ⅵ), Se(Ⅳ), $SeCys_2$, MeSeCys, and SeMet on a Hamilton PRP-X100 anion column with 40mmol/L diammonium hydrogen phosphate (pH=5 with 1% methanol) as the mobile phase and on a ZORBAX SB-Aq C18 reversed-phase column with 10mmol/L citric acid plus 5mmol/L sodium hexane-sulphonate (pH=4 with 1% methanol) as the mobile phase. Secondly, the sample pretreatment conditions were optimized, including the selection of extraction reagents, the amount of extraction reagents, and extraction time. Four extraction reagents (ultrapure water, Tris-HCl buffer solution, proteinase XIV and complex proteinase) were selected for optimization. The effect of proteinase XIV concentration on the extraction was investigated by adding 2, 4, 6, and 8mg/mL proteinase XIV to broccoli samples with selenium content of 0.81mg/kg (calculated as Se). The effect of adding 6mL, 10mL, 12mL, and 15mL of Tris-HCl buffer solution on the extraction was compared. The extraction time of the samples also had a great influence on the extraction efficiency of the selenium speciation. The effects of four extraction times of 1h, 3h, 5h and 7h on the extraction were compared.

RESULTS: The ZORBAX SB-Aq C18 separation system was used in this study because of the short analysis time and high sensitivity of each selenium speciation. Protease XIV was the most effective extraction reagent for selenium; therefore proteinase XIV was chosen as the extraction reagent. The concentration of selenium speciation increased with the concentration of proteinase XIV. The maximum concentration of selenium speciation was reached when the concentration of proteinase XIV was 6mg/mL. It was reported that the use of Tris-HCl buffer solution with proteinase XIV at appropriate pH conditions could further improve the extraction efficiency and maintain the stability of selenium speciation. The volume of Tris-HCl buffer increased, the extraction efficiency of each selenium speciation gradually increased and then decreased, and the final selection of Tris-HCl buffer solution addition was 12mL. A longer extraction time would help to increase the extraction effect, but too long an enzymatic digestion time would also cause a decrease in the stability of SeMet and $SeCys_2$. To ensure high extraction efficiency and reduce the conversion of selenium speciation, an extraction time of 3h was preferred. After optimization and selection, the final analysis method was determined as follows: weighing a certain amount of broccoli sample into 12mL of Tris-HCl (pH=7.4, containing 6mg/mL proteinase XIV) at a concentration of 100mmol/L, vortexing and mixing, and then sonicating at 37℃ for 3h. After centrifugation, the extraction were eluted with 10mmol/L citric acid and 5mmol/L sodium hexane sulfonate (pH=4 with 1% methanol) on ZORBAX SB-Aq C18 reversed-phase column. ICP/MS was used for analysis and determination.

This method can achieve effective separation and determination of five selenium speciation within 8 minutes. The linearity range of the method was 0.3-100.0μg/L, with linear correlation coefficients (r) greater than 0.999. The detection limits of Se(Ⅳ), Se(Ⅵ), MeSeCys, and SeMet were within the range of 1.2-6.0μg/kg (calculated as Se). The standard recovery tests were carried out on broccoli samples at low, medium, and high

concentration. The recoveries of these four selenium speciation, Se(Ⅵ), Se(Ⅳ), MeSeCys and SeMet, were 81.9%-105.3% with relative standard deviations (RSD) less than 5%. The method established in this study was used to determine SeMet in the EU-certified reference material (ERM BC210a, wheat flour), and the measured value of SeMet was within the range of its standard values.

More than 20 commercially available broccoli samples collected from different regions of China were analyzed and determined. The results showed that the selenium speciation in commercially available broccoli was mainly MeSeCys, with small amounts of Se(Ⅵ), Se(Ⅳ), and SeMet, and also a small amount of unknown selenium-containing compounds was also present. Two problems identified in the methodological study were explored. (1) The effect of proteinase XIV dosage on the stability of $SeCys_2$ was investigated by adding 1, 2, 4, and 6mg/mL of proteinase XIV to $SeCys_2$ standard solution, respectively. The results showed that as the concentration of proteinase XIV increased, the signal value of $SeCys_2$ gradually decreased and the signal value of three unknown peaks gradually increased. At the same time, the recovery of $SeCys_2$ in broccoli samples decreased to 10%. Based on the above conditions, it is assumed that the content of proteinase XIV and the matrix of broccoli samples affect the stability of $SeCys_2$.

(2) Three different broccoli samples were selected for Se(Ⅳ) standard recovery tests: fresh commercially available broccoli samples, freeze-dried powder of commercially available broccoli, and freeze-dried powder of broccoli fortified with Se(Ⅳ) selenium fertilizer. A certain amount of the above three samples was added with Se(Ⅳ) standard solution and 100mmol/L Tris-HCl (pH = 7.4, containing 6mg/mL of proteinase XIV). The determination was then carried out according to the proposed analytical method and the mean recoveries of the three samples were found to be 81.1%, 69.5% and 1.53%, respectively. The Kruskal-Wallis rank sum test showed that the recoveries of Se(Ⅳ) were significantly different among the three samples ($p < 0.05$). Previous investigations have found that phenolic substances can affect the stability of Se(Ⅳ) and that the addition of selenium fertilizer during the growth of broccoli can change the phenolics. Based on the above, it is assumed that the presence of phenolics in broccoli samples may affect the determination of Se(Ⅳ).

CONCLUSIONS: A method for the determination of Se(Ⅳ), Se(Ⅵ), MeSeCys, and SeMet in commercially available broccoli by HPLC-ICP-MS is established by selecting and optimizing the sample pretreatment and analytical conditions. The Tris-HCl buffer solution containing proteinase XIV is chosen for the extraction of samples.

It is found that the stability of $SeCys_2$ is affected by the concentration of proteinase XIV and broccoli samples matrix. It is hypothesized that the presence of large amounts of phenolics in the samples can affect the determination of Se(Ⅳ) for reasons to be further explored.

KEY WORDS: broccoli; selenium speciation; high performance liquid chromatography-inductively coupled plasma-mass spectrometry; proteinase XIV

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