

Improved Extraction and Sample Cleanup of Tri-glycoalkaloids α -Solanine and α -Chaconine in Non-denatured Potato Protein Isolates

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Abstract Glycoalkaloids in potato-derived products result in bitter taste and potentially toxic effects at high intakes. Generally, extraction of glycoalkaloids prior to HPLC analysis is carried out by dilute acetic acid. For most potato-derived extracts including heat-coagulated potato proteins, this extraction method is sufficient to achieve satisfying tri-glycoalkaloid (TGA) recoveries. Soluble potato proteins obtained by non-denaturing processes show different requirements for glycoalkaloid extraction. TGA extraction was optimized for two commercially available native potato protein isolates and compared to heat-coagulated potato protein. The highest TGA levels were determined in the extract when extraction was carried out at 40 °C by at least 5 % acetic or propionic acid supplemented with 20 mM Na-1-heptanesulfonate (HSA). Addition of HSA results in substantially improved TGA extraction and induces precipitation of soluble protein which enhanced sample cleanup. On the contrary, extraction of TGA from coagulated potato protein in the presence of HSA showed a reduced TGA extraction efficiency. This improved TGA extraction procedure for soluble non-denatured potato protein isolates results in reliable quantification of bitter tasting and toxic glycoalkaloid levels. This contributes to a non-bitter and safe use of the nutritional and functional benefits of this plant protein in food applications.

Keywords Food safety · Glycoalkaloid · Potato protein · Solanine · Solid-phase extraction

Abbreviations

TGA	Tri-glycoalkaloid
HSA	Na-1-heptanesulfonate
PJ	Potato juice
HMW	High molecular weight potato protein isolate
LMW	Low molecular weight potato protein isolate
kDa	Kilodalton
SPE	Solid-phase extraction
HCl	Hydrochloric acid
HAc	Acetic acid
PPA	Propionic acid
mM	Millimolar

Introduction

Proteins from vegetal sources are gaining more and more interest from the food industry. Today, a wide range of protein isolates from plant sources exists and includes soybean, wheat, rice, pea as well as potatoes. Among these plant proteins, proteins derived from potatoes are considered to have a superior nutritional value. Potato protein is a balanced source of essential amino acids including lysine which is generally deficient in legume and cereal proteins (Knorr 1977; Van Gelder and Vonk 1980). Besides a high nutritional quality, potato protein has also been shown to exhibit good digestibility in both rats and piglets (Nestares et al. 1993; Boisen 1999).

A good source of potato proteins available in large volumes is potato juice (PJ), a by-product from the potato starch industry. In the EU, 7 million tonnes of starch potatoes are produced on a yearly basis (Starch Europe 2014) resulting in a roughly

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same volume of PJ. PJ is composed of minerals, organic acids, glycoalkaloids, amino acids, and phenolic compounds and is relatively rich in soluble protein 15–20 g/l (Lisinska and Leszczynski 1989; Zwijnenberg et al. 2002). Traditionally, potato proteins are extracted from PJ by heat coagulation processes via steam injection causing denaturation and flocculation of the proteins. This results in a nutritional but largely insoluble and non-functional product which finds its way into various feed applications. Alternative methods for purification and fractionation of potato proteins have been studied and include ultra-filtration techniques (Zwijnenberg et al. 2002; Strætkvern and Schwarz 2012); the use of protein precipitation aids such as (in-)organic acids, metal salts, carboxymethyl cellulose, and organic solvents (Knorr 1982; Gonzalez et al. 1991; van Koningsveld et al. 2002; Vikelouda and Kiosseoglou 2004; Bárta et al. 2008; Bártová and Bárta 2009); foam separation technology (Liu et al. 2013); the use of clay minerals (Lindner et al. 1981; Ralla et al. 2012) as well as various (membrane-)chromatographic techniques (Strætkvern et al. 1999; Graf et al. 2009; Løkra et al. 2009; Strætkvern and Schwarz 2012). However, when applied on a larger scale, the use of the majority of these techniques is limited due to fouling, processing costs, or co-purification of the precipitation aid. Besides previous attempts, fractionated potato protein isolates have become commercially available in a food grade quality in recent years (*GRAS Notice 000447: Potato Protein Isolates* 2012). This allows exploitation of nutritional and functional benefits of this plant protein in food applications. Non-denatured potato protein isolates are obtained from PJ via chromatographic isolation techniques and exhibit high solubility and a wide range of techno-functionalities (Alting et al. 2011).

One of the prerequisites for a food-grade potato protein isolate is a final product with sufficiently reduced levels of bitter taste and toxic glycoalkaloids. These secondary plant metabolites are naturally present in *Solanaceae* species like potato, tomato, and eggplant. In potato tubers, two triglycosylated forms of the steroidal alkaloid solanidine exist, α -solanine and α -chaconine. Both tri-glycoalkaloids (TGA) contribute approximately 95 % to the total glycoalkaloid level in potato (Friedman et al. 1997). The total glycoalkaloid level is therefore generally considered as the sum of both triglycosylated forms. The levels of TGA in consumption potatoes generally range 10–100 mg/kg fresh weight and are limited to 200 mg/kg by the US Food and Drug Administration (FDA) (Knuthsen et al. 2009). In contrast, some industrial starch potato varieties can exceed this TGA limit. Large majority of glycoalkaloids in those potatoes remains present in the PJ during starch extraction and tends to partition with proteins in a number of protein extraction methods (Driedger and Sporns 1999; Friedman 2006; Bártová and Bárta 2009). As a result, TGA levels in potato protein isolates can reach levels well over 1000 mg/kg. Ingesting potato protein isolates

or potato products with high levels of glycoalkaloids results in a bitter taste and can cause gastrointestinal disturbances, neurological disorders, and, in some cases, death. The bitter taste becomes apparent at levels of 140 mg/kg TGA or more (Friedman 2006). Morris and Lee (1984) calculated a range of 2 to 5 mg/kg body weight TGA to be the toxic dose for humans while ingestion of 3 to 6 mg/kg could be fatal. To be able to use the benefits of potato protein for human nutrition, it is essential that potato protein isolates are accurately monitored for TGA content.

Quantification of potato glycoalkaloids is generally carried out by HPLC for routine determination as recommended (*AOAC: Official Methods of Analysis 997.13* 2000), although various other techniques have been proven suitable as well (Friedman et al. 1997; Friedman 2004). HPLC analysis of glycoalkaloids is preceded by extraction and sample cleanup. Extraction of TGA has been performed on many if not all parts of the potato plant in either fresh or dried form. Acidic mixtures of water and other polar protic solvents have successfully been used for TGA extraction while non-aqueous solvents were shown to be less suitable for dried samples (Friedman et al. 1997). In a few cases, extraction of TGA was assisted by the addition of an ion-pairing agent (Houben and Brunt 1994). An optimized solid-phase extraction procedure and subsequent HPLC analysis of TGA extracted from coagulated potato protein was reported by Alt et al. (2005). The insoluble nature of coagulated potato proteins allows multiple exhaustive extractions to be performed on a sample with little or no carryover of protein in the solvent. In contrast to coagulated potato proteins, native potato proteins exhibit high solubility in TGA extraction solvents such as dilute organic acid. Consequently, all sample material will be subjected to SPE cartridge with risk of cartridge overloading and poor TGA recoveries as a result. In this study, the extraction of TGA from commercially available denatured and non-denatured (native) potato proteins was optimized for routine quality control analysis. The use of non-aqueous TGA extraction solvents was omitted to avoid necessity of solvent evaporation step before SPE sample cleanup. All three potato protein products show different requirements for an efficient extraction and analysis of TGA content.

Materials and Methods

Materials and Chemicals

The standards of α -solanine and α -chaconine were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Chemicals were purchased from Merck (Darmstadt, Germany) in p.a. quality or LC grade (acetonitrile). Na-1-heptanesulfonate (HPLC grade) and 100 % acetic acid were obtained from VWR BDH Prolabo (Leuven, Belgium).

Ultrapure water was obtained from a MilliQ Academic (Merck-Millipore, Billerica, USA). Potato protein samples were obtained from AVEBE (Veendam, The Netherlands). As a reference for coagulated potato protein, a sample Protamyl PF was used. For the analysis of TGA extraction from non-denatured potato protein, a sample Solanic 206P was taken as a reference for HMW native potato protein and Solanic 306P as a reference for LMW native potato protein. HMW and LMW potato protein samples were selected with relatively high TGA levels and the presence of both α -solanine and α -chaconine.

TGA Extraction

Extraction of TGA was carried out by weighing 100 mg of protein sample in a 50-ml plastic centrifuge tube to which 30 ml TGA extraction buffer was added. Standard TGA extraction buffer was 5 % acetic acid supplemented with 20 mM Na-1-heptanesulfonate unless otherwise stated. Samples were incubated in 40 °C water bath while shaking at 150 strokes/min for 2 h followed by centrifugation at 9000×*g* for a duration of 10 min using a Heraeus Multifuge 1 SR. Supernatant of the extract was filtered using a Pall Acrodisc GHP 0.45- μ m syringe filter. The soluble protein content in these TGA extracts was determined by the use of a Kjeldahl calibrated (Nx6.25) Sprint Protein Analyzer (CEM Corporation, Matthews, USA) using the supernatant of the sample. The molecular weight distribution of the proteins in HMW and LMW potato protein fractions is determined by an Experion method according to the manufacturer's recommendations (Bio-Rad Laboratories, Hercules, USA).

Online SPE-HPLC Method

Analysis was carried out on an isocratic online SPE-HPLC system equipped with a Robothlon online SPE system (Separations Analytical Instruments B.V., Hendrik-Ido-Ambacht, The Netherlands) coupled to a Smartline HPLC pump 1000 (Knauer, Berlin, Germany), Smartline variable single wavelength UV/VIS detector 2520 (Knauer, Berlin, Germany), a Jetstream 2 plus column thermostat (Industrial Electronics, Langenzersdorf, Austria), a Model 2003 vacuum degasser (Biotech, Onsala, Sweden), and a Chromgate v.3.3.2 data system (Knauer, Berlin, Germany). The online SPE system contains an Oasis HLB Prospect-2/Symbiosis 10 × 2mm cartridge (Waters, Milford, MA, USA). Cartridges are replaced preventively every 96 cycles. The cartridge is washed at a flow rate of 1 ml/min for 4 min with 100 % acetonitrile followed by a 3-min wash with ultrapure water after which an 850 μ l quantity of a TGA extract was loaded onto the cartridge. The cartridge was subsequently washed for 30 s with water, 2 min with wash buffer 1 (25 % acetonitrile/1.5 % NH₄OH), and 30 s with ultrapure water followed by 4 min

with wash buffer 2 (15 % acetonitrile/10 mM phosphate buffer pH .6). TGA was retrieved from the cartridge by the mobile phase of the HPLC system. Separation of α -solanine and α -chaconine was carried out on a Hypersil ODS 250 mm × 4.6 mm 5 μ m C18 column with a Hypersil ODS C18 5.0 × 4.6 mm guard column (Thermo scientific, Waltham, USA) at a temperature of 34 °C and a flow rate of 1.6 ml/min. The mobile phase consisted of a 60/40 mixture of 100 % acetonitrile and 10 mM phosphate buffer pH 7.6 degassed using the degas function of a ultrasonic cleaner (VWR International, Leuven, Belgium) and filtered over a 0.45- μ m PVDF filter (Waters, Milford, MA, USA). Chromatograms were recorded at a wavelength of 202 nm.

Recovery

The effect of protein concentration during TGA extraction was determined by preparing a 16.6 mg/ml solution of HMW and LMW potato protein separately in TGA extraction buffer. This was diluted in eight steps with TGA extraction buffer to cover a range of protein concentration down to 1.7 mg/ml followed by TGA extraction and online SPE-HPLC analysis. TGA content in gram per kilogram was plotted against the protein concentration during TGA extraction to show equal recoveries at 3.3 mg/ml protein or less. Recovery of the calibration curve in sample matrix was carried out by extracting 0.1 g of HMW and LMW potato protein separately in 30 ml TGA extraction buffer. To cover the same concentration range as the calibration curve, α -solanine and α -chaconine standards were added. TGA extraction and online SPE-HPLC analysis was carried out as described above. Recovery of the calibration line in LMW and HMW matrices is represented by the coefficient of the curve.

Results and Discussion

TGA Extraction of Heat-Coagulated Potato Protein

TGA extraction of three potato protein isolates was tested using a series of extraction solvents. The potato protein isolates include an insoluble protein obtained by heat coagulation of potato juice (CPP) and two non-denatured protein isolates obtained by chromatographic separation and isolation of potato proteins from potato juice. Fractionation of soluble potato proteins results in a fraction which is composed of proteins with a low molecular weight of less than 35 kDa (LMW) and another fraction of proteins with a high(er) molecular weight above 35 kDa (HMW). Heat-coagulated potato proteins naturally contain levels of TGA well over 1000 mg/kg due to partitioning of the alkaloids with the protein in the coagulation process. TGA extraction efficiency was evaluated by acidic aqueous TGA extraction solvents containing organic and

inorganic acids. Good solubility of the alkaloids requires an acidic environment (Wolf and Duggar 1946) while the use of organic acids provides a reduced polar environment with high TGA extraction efficiency (Friedman et al. 1997). Organic acid solvents were supplemented by the addition of the ion-pair agent sodium-1-heptanesulfonate (Houben and Brunt 1994). Variation in the extraction of TGA between the three different potato protein products was observed (Fig. 1). TGA levels in the extracts obtained by 5 % acetic acid supplemented with 20 mM Na-1-heptanesulfonate (HSA) were normalized to 100 % for all three potato protein isolates. Extraction of TGA from heat-coagulated potato proteins was equally efficient with all three solvents independent of the concentration. The presence of 20 mM HSA resulted in a reduced level of TGA of this protein sample. The ratio α -solanine/ α -chaconine was 1:1 for all extracts showing no preferred extraction of either solanine or chaconine in any of those extracts (Table 1).

TGA Extraction of Native Potato Protein Isolates

Extraction of TGA from HMW potato protein was equally efficient in the presence of 10–50 mM hydrochloric acid as extraction with 5 or 8 % of either acetic acid or propionic acid. Reduced TGA levels observed in extracts of 2 % organic acid could be attributed to a reduced extraction of α -solanine as

indicated by a low α -solanine/ α -chaconine ratio (Table 1). Addition of 20 mM HSA to acetic acid or propionic acid did not result in the detection of elevated TGA levels in the extract. In contrast to HMW potato protein, extraction of TGA from non-denatured LMW potato protein by various extraction buffers resulted large differences. Higher TGA levels were determined in the extract at increasing acid concentration. Propionic acid was more efficient in extracting TGA compared to acetic acid or dilute levels of hydrochloric acid. Extraction of α -chaconine is marginally dependent of the acid concentration while the proportion α -solanine in the extract increased at increasing acid concentration. The reduced polarity of propionic acid over acetic acid or hydrochloric acid extraction solvents enhances the partitioning of TGA in the aqueous phase. This resulted in the quantification of higher TGA levels in the LMW protein product (Table 1 and Fig. 2a). High retention of specifically α -solanine on the LMW potato protein fraction suggests the presence of a solatriose-specific binding protein in the LMW potato protein fraction. Two sugar moieties that differ solatriose from chacotriose are β -D-glucopyranosyl and β -D-galactopyranosyl residues. TGA extraction of LMW potato protein in the presence of 2 % acetic acid supplemented with either 50 mM D-glucose or D-galactose did not improve extraction of α -solanine (data not shown). These observations suggest high affinity of α -solanine for native LMW potato protein is not

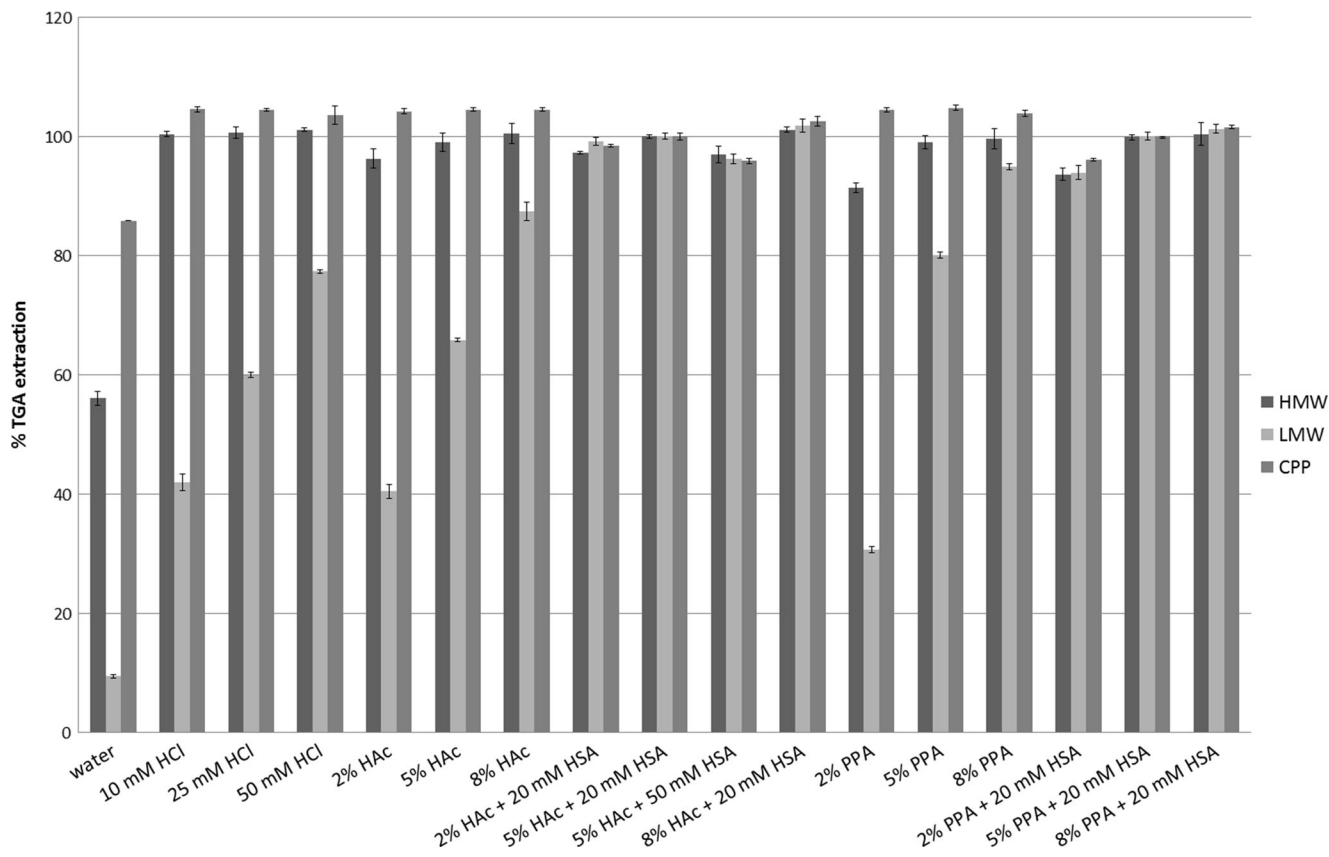


Fig. 1 Average glycoalkaloid extraction efficiency of native HMW and LMW potato proteins and coagulated potato protein (CPP) by various extraction buffers normalized to TGA extraction by 5 % HAC + 20 mM HSA ($n = 3$)

Table 1 Average TGA levels (mg/kg) and ratio α -solanine/ α -chaconine in HMW, LMW, and coagulated potato protein (CPP)

Extraction buffer	HMW			LMW			CPP		
	TGA	sol/chac	grp	TGA	sol/chac	grp	TGA	sol/chac	grp
water	251	N/A	B	66	N/A	B	1796	1.1	B
10 mM HCl	450	4.3	A	296	3.2	B	2188	1.1	C
25 mM HCl	451	4.4	A	423	4.7	B	2187	1.1	C
50 mM HCl	453	4.2	A	545	5.7	B	2168	1.1	C
2 % HAc	432	3.8	B	285	2.7	B	2181	1.1	C
5 % HAc	444	4.3	A	464	4.1	B	2187	1.1	C
8 % HAc	450	4.3	A	616	5.5	B	2186	1.1	C
2 % HAc + 20 mM HSA	436	4.3	A	699	6.6	A	2060	1.1	B
5 % HAc + 20 mM HSA	448	4.2	A	705	6.7	A	2093	1.1	A
5 % HAc + 50 mM HSA	434	4.4	B	678	6.6	B	2006	1.1	B
8 % HAc + 20 mM HSA	453	4.2	A	718	6.5	A	2145	1.1	C
2 % PPA	410	3.8	B	216	2.3	B	2186	1.1	C
5 % PPA	444	4.0	A	564	5.3	B	2193	1.1	C
8 % PPA	447	4.0	A	669	6.1	B	2173	1.1	C
2 % PPA + 20 mM HSA	420	4.2	B	662	6.5	B	2009	1.1	B
5 % PPA + 20 mM HSA	448	4.4	A	706	6.5	A	2089	1.1	A
8 % PPA + 20 mM HSA	450	4.4	A	714	6.5	A	2125	1.1	C

One-way ANOVA grouping of the TGA content versus the standard indicated by A (identical to standard extraction), B (lower than standard extraction), or C (higher than standard extraction). Standardized extraction is indicated in italic

N/A not applicable

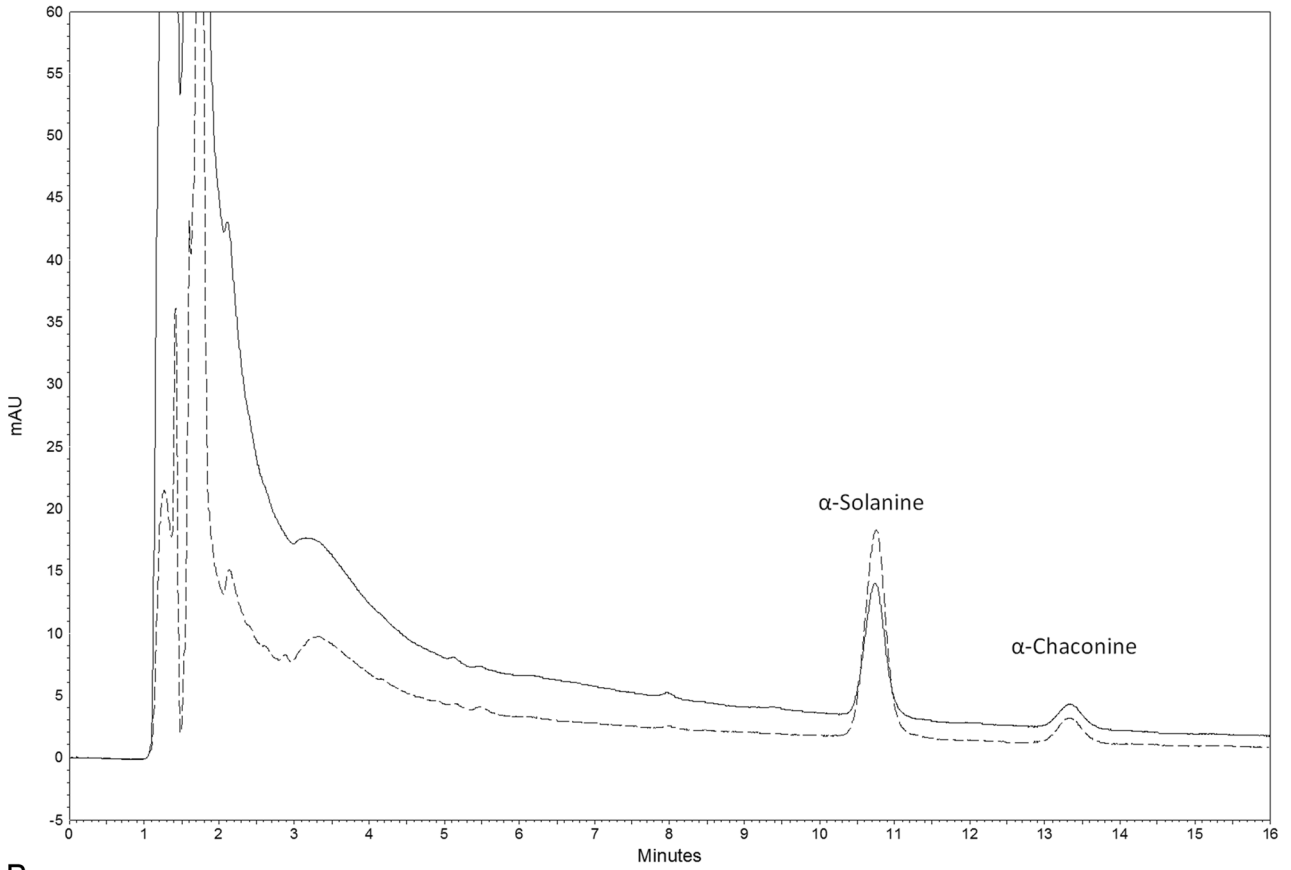
based on a direct sugar-specific interaction with proteins such as lectins. Ion-pair extraction using 20 mM HSA supplemented to both organic acids at either 5 or 8 % concentration resulted further enhanced extraction of α -solanine for the LMW potato protein fraction. Formation of an ion-pair of HSA with the amine group in the solanidine aglycon is likely to facilitate extraction of α -solanine in this potato protein fraction. The exact nature of this solatriose-specific interaction between α -solanine and native LMW potato protein fraction remains however unclear. Enhanced extraction of α -solanine in the presence of HSA might also be a result of the observed protein precipitation induced by the ion-pairing agent. Changes in the folded state of the LMW protein can alter the binding kinetics of α -solanine to a specific domain resulting in an improved extraction efficiency. HSA-induced protein precipitation of both HMW and LMW proteins in the extract was enhanced at temperatures up to 40 °C. TGA extraction carried out at temperatures above 40 °C does not result in a further increase protein precipitation nor TGA extraction (data not shown). Residual protein levels in the TGA extract after centrifugation and filtration are less than 10 % of the original quantity introduced in the extraction. Protein precipitation in LMW as well as HMW samples by HSA at 40 °C reduced the interference of the matrix in the chromatogram (Fig. 2a, b). Heat-coagulated potato proteins do not benefit from the addition of HSA as protein precipitation aid as a result of the insoluble nature of the product.

Reduced TGA content after extraction in the presence of 50 mM HSA was observed for all three potato protein samples (Fig. 1). Extraction of TGA from LMW protein is enhanced by the presence of HSA. The optimal HSA concentration for TGA extraction was determined. LMW potato protein incubated with 5 % HAc in the presence of 0 up to 100 mM HSA at 40 °C revealed that the highest TGA levels were determined when extraction solvent was supplemented with 10 or 20 mM HSA (Fig. 3). Higher HSA levels during extraction result in rapid formation of a protein precipitate which limits the extraction of TGA.

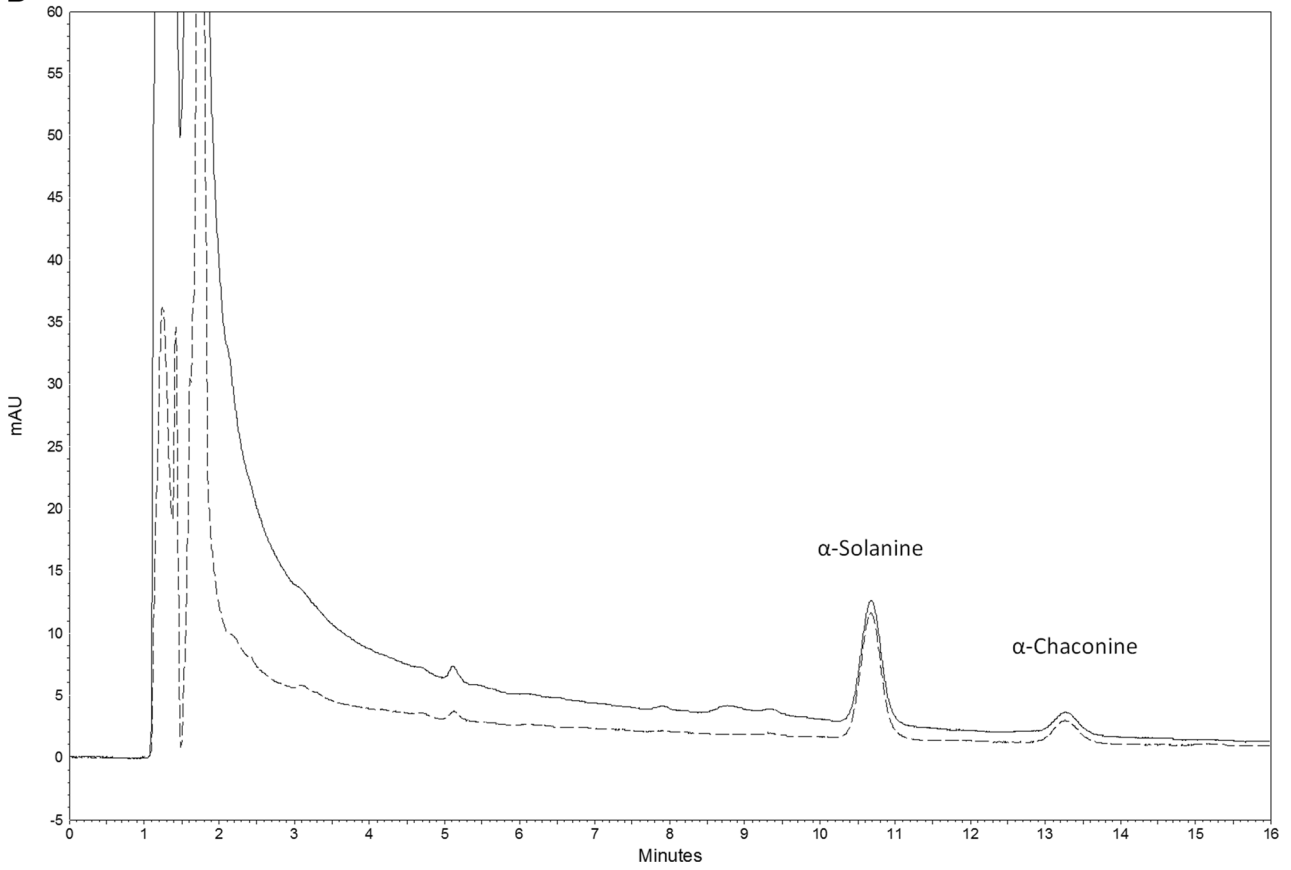
Recovery of TGA

High solubility of non-denatured potato protein in TGA extraction buffer affects its recovery on the SPE cartridge. TGA recovery of both α -solanine and α -chaconine was determined in a HMW as well as a LMW potato protein matrix. Protein fractions were extracted with 5 % acetic acid and 20 mM HSA supplemented with α -solanine and α -chaconine. A serial dilution of the extract in the acetic acid-HSA extraction buffer covers a range of protein concentration of 0.05–0.5 g/30 ml followed by a 2-h extraction at 40 °C. Quantification of the samples after SPE and HPLC analysis revealed that over 97 % of both α -solanine and α -chaconine could be recovered up to a concentration of 0.1 g protein per 30 ml extraction buffer. Higher levels

A



B



◀ **Fig. 2** The effect of 20 mM HSA supplementation (*dashed line*) to 5 % acetic acid on the chromatograms of TGA extracts obtained from LMW potato protein (a) and HMW potato protein (b)

of protein in the extraction buffer resulted in a diminished recovery of TGA. Recoveries of 89 % (HMW) and 91 % (LMW) were obtained in case extraction was carried out at a protein concentration of 0.5 g/30 ml TGA extraction buffer.

Recovery of the calibration curve in a sample matrix at 0.1 g HMW or LMW potato protein was 95.6 % for α -solanine and 95.5 % for α -chaconine in case of HMW potato protein and 97.8 % for α -solanine and 96.3 % for α -chaconine in case of LMW potato protein. Analysis of 0.5 μ g/ml ($N = 43$) and 4 μ g/ml ($N = 45$) solanine and chaconine standards as well as a LMW protein TGA extract ($N = 55$) over a period of 5 months resulted in relative standard deviation of 2.22/4.29 %, 2.12/1.97 %, and 2.94/3.54 % for α -solanine and α -chaconine, respectively.

Limit of Quantification

TGA levels in commercially available native potato protein isolates HMW and LMW are less than 300 mg/kg, and for a number of applications, an upper limit of 150 mg/kg is preferred. Reducing the sample quantity to 100 mg in 30 ml TGA extraction buffer in order to maintain a recovery of at least 95 % affects the quantification limit of the system. Reliable quantification of both α -solanine and α -chaconine could be achieved down to 0.125 μ g/ml concentrations referring to a limit of quantification of 37.5 mg/kg α -solanine or α -chaconine.

Increased TGA extraction volumes from coagulated potato proteins can be applied to SPE sample cleanup due to the

Fig. 3 Extraction of TGA from LMW potato protein with 5 % acetic acid supplemented by HSA at 0–100 mM concentrations. Data normalized to 20 mM HSA

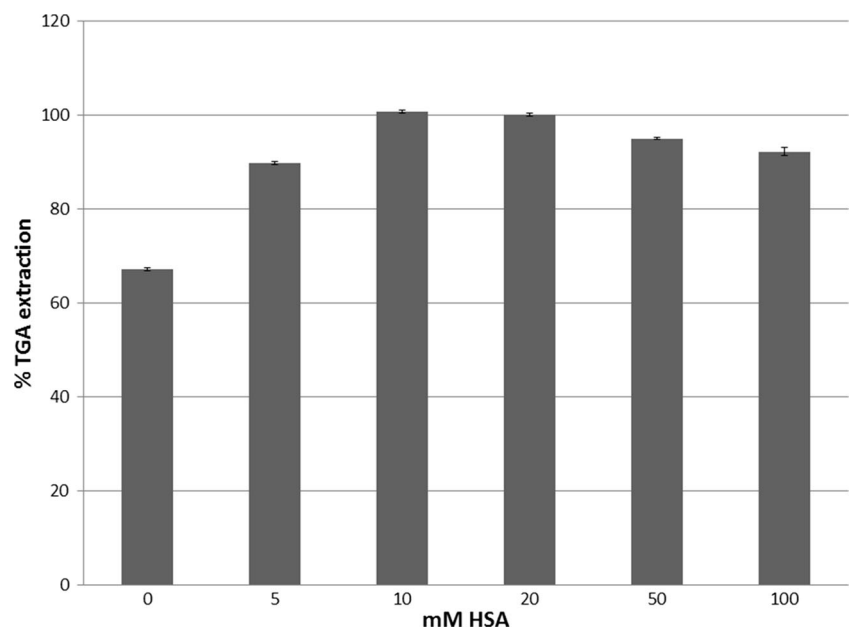


Table 2 α -Solanine and α -chaconine levels in commercial batches determined by applying 1 and 5 volumes of TGA extract to the SPE cartridge

Sample	SPE volumes	α -Solanine		α -Chaconine	
		mg/kg	std	mg/kg	std
HMW	1	76.2	3.3	N/D	
(206P9020 ^a)	5	78.6	0.5	14.8	0.2
LMW	1	81.3	2.8	N/D	
(306P0010 ^a)	5	85.1	2.9	9.4	0.6

N/D not detected

^a Commercial batch numbers

insoluble nature of the proteins. This results in an increased signal and lower detection limit (Alt et al. 2005). Similar to TGA extracts from insoluble coagulated potato proteins, precipitation of soluble potato proteins induced by HSA during TGA extraction allows the application of larger volumes to the SPE cartridge. Applying 1, 2, and up to 5 volumes of TGA standards to the SPE cartridge results in a linear increase in peak area for both α -solanine and α -chaconine. Increasing the volume of the TGA extract applied to the SPE cartridge by a factor 5 allows reliable quantification of TGA levels which remain below detection limit at 1 volume (Table 2). This results in an improved detection of the actual TGA level in the sample down to levels of 7.5 mg/kg.

Conclusions

Efficient extraction of TGA from a native potato protein isolate is determined by the type of extraction

solvent, concentration, presence of Na-1-heptanesulfonate (HSA), and the amount of protein in the extraction buffer. High recoveries of TGA from native HMW and LMW potato protein fractions were obtained using a TGA extraction solvent composed of at least a 5 % concentration of either acetic acid or propionic acid in water. Ion-pair extraction using 20 mM HSA improves the recovery of α -solanine from the LMW potato protein fraction resulting in an enhanced accuracy of the actual TGA level in this product. Furthermore, HSA serves as a protein precipitation aid for both HMW and LMW potato proteins. HSA-induced protein precipitation is enhanced at extraction temperatures up to 40 °C resulting in chromatograms with fewer impurities and improved baseline. Overloading of the cartridge by an excess of soluble protein in the TGA extract is prevented and larger volumes can be applied to the SPE cartridge. This contributes to a detection limit of the system of less than 10 mg/kg and recoveries over 97 % were obtained. Addition of HSA to the extraction buffer diminished the recovery of TGA from coagulated potato protein samples. The use of this improved TGA extraction procedure for denatured and non-denatured potato protein isolates allows accurate detection of low TGA levels through routine quality control analysis.

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Compliance with Ethical Standards

Conflict of Interest Marc C. Laus declares that he has no conflict of interest. Gert Klip declares that he has no conflict of interest. Marco L. F. Giuseppin declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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