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Identification of *Smallanthus sonchifolius* in Herbal Tea Mixtures by PCR and DART/TOF-MS Methods

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Abstract

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The identification of yacon, a medicinal plant, in tea mixtures by rapid Polymerase Chain Reaction (PCR) and the Direct Analysis in Real Time coupled with Time-of-Flight Mass Spectrometry (DART/TOF-MS) method were evaluated. Three tea products and a pure yacon tea were analysed using the molecular method PCR, concretely the intraspecific variation of the internal transcribed spacer (ITS) regions of rDNA and the DART method coupled with TOF-MS. The results show the reliability of PCR and restriction cleavage of the ITS as a combined approach to confirm the presence of yacon in herbal tea mixtures. Three fragments of approximately 700, 408, and 235 bp in length are present when yacon is a part of the herbal tea mixture. The Principal Component Analysis (PCA) based on the fingerprints of the complete Total Ion Current (TIC) mass spectra shows sufficient separation of herbal teas with and without yacon leaves. The reported methods are technically rapid and can be used as an effective tool for the purposes of yacon identification or authentication.

Keywords: authentication; DraIII; ITS; yacon

Yacon [*Smallanthus sonchifolius* (Poepp. et Endl.) H. Robinson, Asteraceae] is a native plant to the Andes and it represents a traditional crop of Bolivia, Ecuador, and Peru still used in traditional medicine. Yacon tubers contain fructooligosaccharides of inulin type β (2→1) which are known for their ability to keep the colon healthy. The sweetness of yacon is predominantly caused by fructose, which is by some 70% sweeter than sucrose. It is recommended that diabetics and persons suffering from digestive problems consume yacon because its carbohydrates are not available for absorption by the small intestine (Lachman *et al*. 2003). Additionally, yacon leaves contain important compounds including phenolics, catechol, terpenes, and flavonoids (Lachman *et al*. 2003). The health benefit analysis of using yacon leaves in teas has been widely reported (Vilhena *et al*. 2000; Aybar *et al*. 2001; Genta *et al*. 2010). The fact that this crop has antidiabetic, nutritious, and fertility enhancing properties changes the view and increases the economic value of this crop in European conditions (Fukai *et al*. 1993; Plchová 1997; Fernández *et al*. 2006). Agronomic research on yacon confirms the possibility of successful yacon cultivation in central Europe (Fernández *et al*. 2006, 2013).

It is important nowadays to develop not only cultivation characteristics for underutilised crops and species, but also methods need to be developed to identify, to distinguish, or to authenticate this species and its

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products by working with molecular and DNA markers (Random Amplified Polymorphic DNA – RAPD; Inter-Simple Sequence Repeat – ISSR; Amplified Fragment Length Polymorphism – AFLP, or Inter-Primer Binding Site Polymorphism – iPBS) as well as a Direct Analysis in Real Time (DART) technique. Internal transcribed spacer (ITS) sequence analysis has shown a potential to identify yacon not only among other plants, but also among other members of the yacon genus (Žiarovská *et al*. 2013, 2014). The DART technique is used for the inspection of food, especially the determination of pesticides, fatty acids, and cholesterol, and for assessing the quality and authenticity of soft drinks, olive oils, mustards (Čajka *et al*. 2008; Václavík *et al*. 2009; Alballa *et al*. 2014; Prchalová *et al*. 2014). The DART technique has been used for monitoring tea fermentation and manufacturing (Fraser *et al*. 2013), for the determination of anisatin in teas (Shen *et al*. 2012), for quality assessment of Basha herbal tea (Deng & Yang 2013). This is a relatively new ionisation technique of mass spectrometry, however its principles and advantages are well described in the literature (Chen *et al*. 2009; Hajslová *et al*. 2011; Huang *et al*. 2011).

In the present study, molecular based and spectrometry techniques were developed for the detection and identification of *Smallanthus sonchifolius* in tea mixtures by Polymerase Chain Reaction (PCR) based amplification of the ITS of all species in a tea mixture by universal primers, and subsequently yacon specific cleavage of the amplified PCR products and through DART coupled with a Time-of-Flight Mass Spectrometry (TOF-MS) technique.

MATERIAL AND METHODS

Tea mixtures and reference sample for PCR and DART/TOF-MS. In total, three tea products and a pure yacon tea were used in the analysis. Individual tea mixtures were chosen from local stores in the following manner: firstly, each tea product contained at least five different plant species and secondly, each tea contained at least one plant from the subclass Asteridae (Table 1).

PCR and amplified product analysis. For the PCR reactions yacon tea mixtures were prepared by mixing 2 g of the teas from Table 1 together with 2 g of pure yacon tea. The tea mixtures without yacon were labelled as 3A, 6A, 9A and the tea mixtures containing yacon were labelled as 3B, 6B, and 9B (Table 1). Pure yacon tea was labelled as 4. The

Table 1. Characteristics of tea mixtures used in the analysis

Code of mixture	Plant species in the mixture	Family	Belonging to Asteridae
3A	*Cichorium intybus*	Cichoriaceae	+
	Aspalathus linearis	Fabaceae	–
	Rosa canina	Rosaceae	–
	Zingiber officinale	Zingiberaceae	–
	Glycirrhiza glabra	Fabaceae	–
3B	*Cichorium intybus*	Cichoriaceae	+
	Aspalathus linearis	Fabaceae	–
	Rosa canina	Rosaceae	–
	Zingiber officinale	Zingiberaceae	–
	Glycirrhiza glabra	Fabaceae	–
	Smallanthus sonchifolius	Asteraceae	+
6A	*Matricaria recutita*	Asteraceae	+
	Thymus vulgaris	Lamiaceae	+
	Primula veris	Primulaceae	–
	Malva mauritiana	Malvaceae	–
	Althea officinalis	Malvaceae	–
	Plantago lanceolata	Plantaginaceae	–
	Achillea millefolium	Asteraceae	+
6B	*Matricaria recutita*	Asteraceae	+
	Thymus vulgaris	Lamiaceae	+
	Primula veris	Primulaceae	–
	Malva mauritiana	Malvaceae	–
	Althea officinalis	Malvaceae	–
	Plantago lanceolata	Plantaginaceae	–
	Achillea millefolium	Asteraceae	+
	Smallanthus sonchifolius	Asteraceae	+
9A	*Thymus vulgaris*	Lamiaceae	+
	Tilia cordata	Malvaceae	–
	Foeniculum vulgare	Apiaceae	–
	Polygonium vulgare	Polygonaceae	–
	Pimpinella anisum	Apiaceae	–
	Glycirrhiza glabra	Fabaceae	–
9B	*Thymus vulgaris*	Lamiaceae	+
	Tilia cordata	Malvaceae	–
	Foeniculum vulgare	Apiaceae	–
	Polygonium vulgare	Polygonaceae	–
	Pimpinella anisum	Apiaceae	–
	Glycirrhiza glabra	Fabaceae	–
	Smallanthus sonchifolius	Asteraceae	+
4	*Smallanthus sonchifolius*	Asteraceae	+

Tea mixtures without yacon were labelled as 3A, 6A, 9A; tea mixtures containing yacon were labelled as 3B, 6B, 9B; pure yacon tea was labelled as 4

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primers used for the amplification of the ITS region were those reported by White *et al.* (1990). The amplified ITS region consists of three parts: ITS1, ITS2, and the highly conserved 5.8S rDNA exon located in-between. Both of the primers were used for the universal PCR and sequencing reactions. The sequences of primers were as follows: the forward primer 5'TCCGTAGGTGAACCTGCGG3' and reverse primer 5'TCCTCCGCTTATTGATATGC3'. Individual amplifications of the ITS were performed in 1X buffer containing 20 mm $MgCl_2$, 0.2 mm dNTPs, 300 nm forward and reverse primer, and 1U of MyTaq DNA polymerase (Bioline, London, UK) in a total volume of 15 µl. The cycling profile was as follows: 95°C, 3 min [95°C, 40 s; 52°C, 40 s; 72°C, 40 s] 34 ×, 72°C, 7 minutes.

The amplified PCR products were separated on 1.2% agarose gels along with a 100 bp DNA ladder (ThermoScientific, Waltham, USA) to confirm the specificity of the PCR. The products which resulted from restriction cleavage were separated by an Experion Automated Electrophoresis System (BioRad, Hercules, USA) using the Experion™ DNA 1K Analysis Kit for precise determination of the restriction fragment length and by 2% agarose gel electrophoresis, respectively. These two separating methods were performed to adapt this method to labs depending on the availability of equipment. For both analyses (PCR and restriction cleavage) the reference yacon tea was used to confirm the specificity of yacon identification.

Database searching and restriction cleavage site prediction. The National Centre for Biotechnology Information (NCBI) database was searched for existing ITS nucleotide data on the individual plant species contained in the analysed herbal tea mixtures. All ITS data found for each individual plant used in the herbal tea mixtures were transformed into Fasta format and analysed by ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2/) against themselves to inspect their similarity, in particular for the partial sequences that were found. Finally, for each plant in the herbal tea mixtures belonging to the order Asterales, the complete ITS was chosen (Table 2) for the final alignment in the MultiAlin software (multalin.toulouse.inra.fr/multalin/), where the specificity for the restriction cleavage of yacon only was analysed.

Restriction cleavage was performed based on the yacon specific ITS nucleotide sequence as reported by Žiarovská *et al.* (2013) and stored in NCBI as KF826287. Restriction endonuclease DraIII (New England Biolabs® Inc., Ipswich, USA) was used for restriction analysis and the presence of yacon was confirmed by obtaining PCR products via the following protocol: 10 µl of PCR product, 3 µl of 10NEB4 buffer, 0.5 µl of DraIII enzyme, and 16.5 µl water. Restriction digestion was performed in a total volume of 20 µl.

Table 2. Sequences of the ITS compared with their matches

Plant	Accession in NCBI	Length (nt)
Smallanthus sonchifolius	KF826287	643
Cichorium intybus	JQ230974	719
Matricaria recutita	EU179212	669
Achillea millefolium	AY603185	623
Thymus vulgaris	EU785939	671

DART/TOF-MS method. DART analyses were performed according to Prchalová *et al.* (2014). The analysis was performed employing a DART SVP 100 ion source (IonSense, Saugus, USA) coupled to a TOF LC-MS 6224 mass spectrometer (Agilent Technologies, Santa Clara, USA) through a vacuum interface (IonSense, USA) represented by a ceramic tube 3.18 mm i.d. and 83 mm in length (for the DART-100 ion source). Deep vacuum in TOF was created by an Edwards E2M28 vacuum pump (Edwards, Crawley, UK). Standards and samples were introduced into the ionisation region using a 12-DIP-it autosampler (IonSense, USA). The DART ion source was operated in a positive and negative mode with helium reaction gas (purity 4.8; SIAD, Prague, Czech Republic) under the following conditions: flow rate of 3.5 l/min, grid voltage 350 V, autosampler velocity 1 mm/s, and ionisation gas temperature of 350 or 450°C. TOF-MS was run in a positive mode; the fragmentor and the skimmer potentials were 175 and 65 V, respectively. The sample was taken with glass DIP-it rods (IonSense, USA). For data acquisition and processing, the Agilent MassHunter Workstation Acquisition B.04.00 and Agilent MassHunter Workstation Software Qualitative B.04.00 (both Agilent Technologies, USA) were used. For mass spectra studies, the total ion current (TIC) chronogram was registered in the range of *m/z* 100–1500. DART was controlled by DART-SVP 3.0.3b software (IonSense, USA). Tuning of TOF-MS was carried out before each set of samples using an API-TOF Reference Mass Solution Kit (Agilent Technologies, USA).

Statistical analysis and data from the DART/TOF-MS method. Data tests were triplicated for each sample and mean values ± SD (standard deviation)

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are reported. Differences at $P < 0.05$ were regarded as significant. All statistical analyses, including PCA, were performed using Statistica 10 (StatSoft CR, Prague, Czech Republic) and Excel 2010 (Microsoft, Prague, Czech Republic). The MassHunter software enables the export of mass data and spectra into Excel (Microsoft, Czech Republic). In Excel (Microsoft, Czech Republic) a macro was created which enabled sorting of the data according to *m/z* for a given abundance threshold. Data of the DART/TOF-MS method were statistically processed by the principal component analysis (PCA) based on the fingerprints of the complete TIC mass spectra.

RESULTS AND DISCUSSION

In accordance with the aim of the present study, three tea mixtures were analysed by restriction cleavage and by spectrometry technique to confirm the potential of the presented methods to authenticate the presence of yacon in herbal tea mixtures.

Specificity of ITS amplification in herbal tea mixtures. In the performed PCR, the size variations of the ITS regions which were observed was fully predicted based on the ITS length of each individual plant part in the herbal mixtures. All ITS sequences for all species were obtained from the NCBI database. The concrete range of the amplified ITS regions was from 643 bp up to 705 bp. In this study, universal ITS primers were used which were designed by White *et al.* (1990) to generate amplicons that corresponded to the internal transcribed spacers of all plant species used in the tested herbal tea mixtures. Serial dilutions of genomic DNA extracted from the herbal tea mixtures were tested to assess the reliability and repeatability of the PCR assay or developed methodology. The stability of ITS amplification in the reported conditions was confirmed by varying the amount of DNA from 12.5–50 ng.

Development of a yacon identification system based on restriction analysis. The DraIII restriction

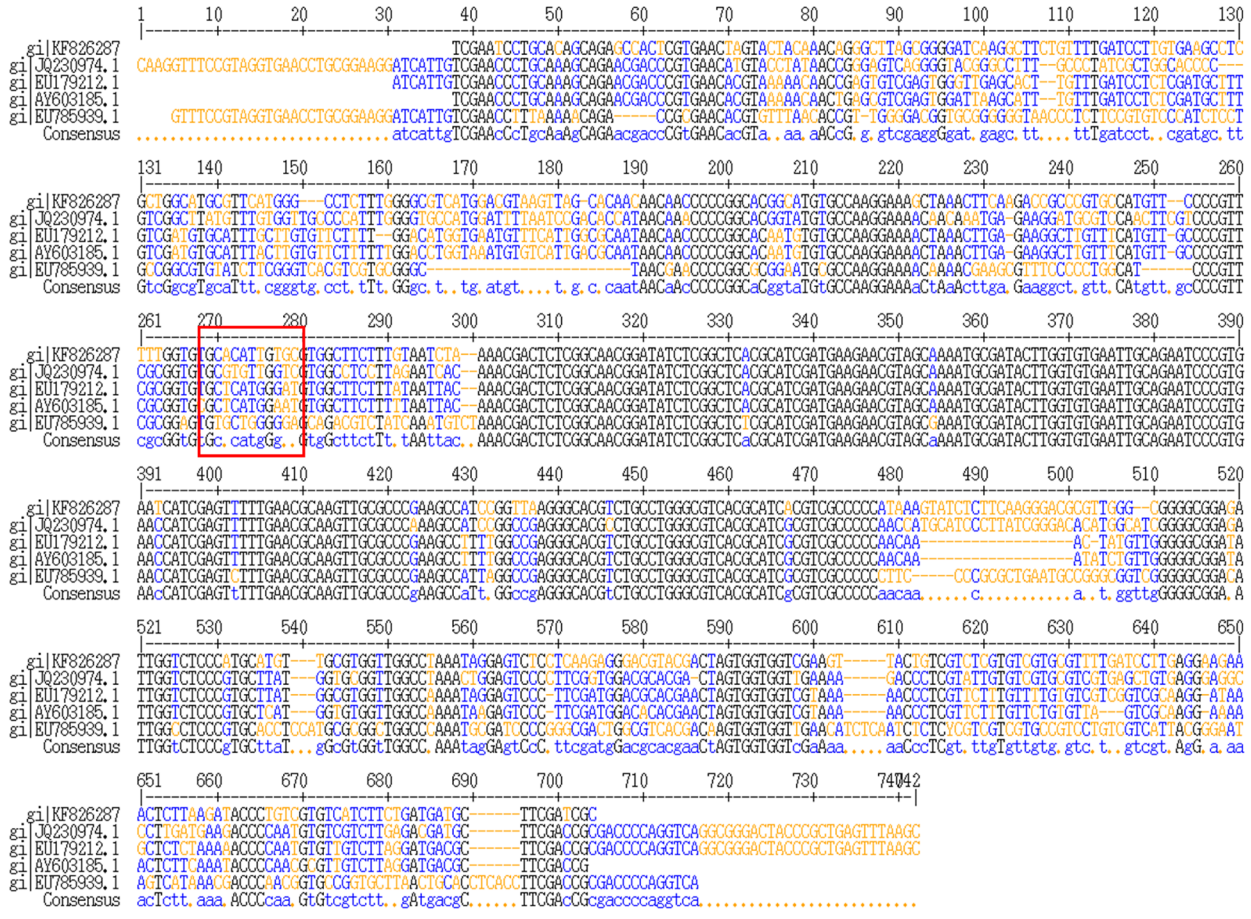

Figure 1. Comparison of internal transcribed spacer (ITS) sequences of the Asteridae in the analysed herbal tea mixtures as performed in the MultiAlin software

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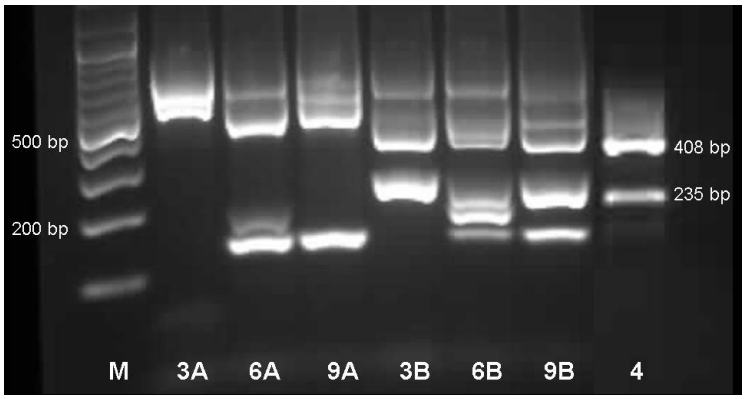

Figure 2. Agarose gel electrophoresis for the restriction cleavage of herbal tea mixtures without (3A, 6A, 9A) and with yacon (3B, 6B, 9B), and comparison with the pure yacon tea (4); the used samples are described in Table 1

enzyme was used to cleave the obtained PCR amplicons due to its ability to generate specific restriction profiles when yacon is present in the herbal tea mixture. The recognition sequence site for DraIII restriction (GTGCGT) is typical of the nucleotides from 232 to 237 in the ITS of *Smallanthus sonchifolius* (NCBI accession KF826287; Žiarovská *et al*. 2013, 2014) and is also unique within the corresponding sequences analysed in plant species belonging to the Asteridae – *Cichorium intybus* (NCBI accession JQ230974), *Matricaria recutita* (NCBI accession EU179212), *Thymus vulgaris* (NCBI accession EU785939), and *Achillea millefolium* (NCBI accession AY603185) as is shown in Figure 1.

Based on the expected restriction cleavage, one to three fragments are visible in the herbal tea mixtures without yacon (Figure 2). Two yacon specific fragments plus fragments corresponding to those from the herbal tea mixtures are visible in teas containing yacon. Two pure fragments of 235 bp and 408 bp in length are present in the pure yacon tea.

Restriction cleavage in herbal tea mixtures specific to yacon. The fragments generated in the digestion have matched in all cases (herbal tea mixtures with or without yacon and pure yacon tea) with the expected ones on the basis of the predicted restriction map. The restriction profiles were analysed by both agarose gel electrophoresis and capillary electrophoresis. When agarose gel electrophoresis (Figure 2) is used to analyse the results of restriction cleavage, only the predicted fragments are visible. As to the much higher sensitivity of capillary electrophoresis, in addition to the predicted fragments (Figure 3), weak fragments are also visible on the generated virtual electrophoregram. These are so weak that agarose gel electrophoresis is not sensitive enough to detect them. These fragments are supposed to be the products of partial primer annealing, the result of the variability of the DNA of the different plant species which were used in the analysis. However, in all of the analysed samples, the main predicted fragments of the correct corresponding length were the most visible.

When comparing the samples 3B, 6B, and 9B which represent herbal tea mixtures with the addition of yacon with the cleavage results of pure yacon tea, two separated restriction fragments corresponding to those in the pure yacon sample are confirmed. This indicates that the data presented here for the purposes of yacon identification in the tea mixtures is highly sensitive.

Yacon is widely used as a part of tea mixtures. In Brazil, dried leaves are used to prepare a medicinal tea with antidiabetic properties that are attributed to yacon leaves (Vilhena *et al*. 2000). Dried yacon leaves are used in Japan, where they are mixed with common tea leaves. Based on these findings, a PCR based method is very practical for yacon identification in the tea mixtures. The molecular technology that is presented in this

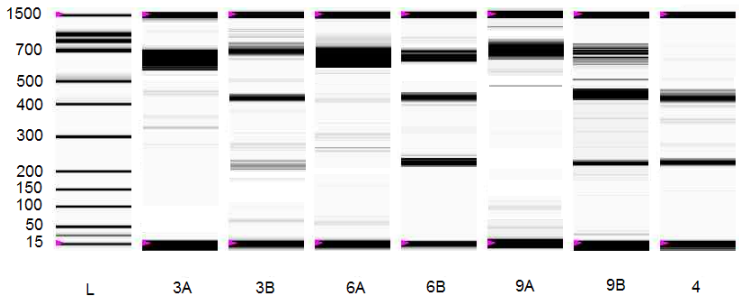

Figure 3. Virtual electrophoregram based on capillary electrophoresis for the analysed samples; the used samples are described in Table 1

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study can be adapted and performed by laboratories with different, basic, or advanced equipment.

Internal transcribed spacers are reported in the literature as belonging among the most informative regions of variable DNA used for phylogenetic analysis to distinguish relationships within genera at the species level (Clements 2003; Jorgensen *et al.* 2003). The nucleotide sequence of the yacon ITS that was reported previously by Žiarovská *et al.* (2013) describes the specific nucleotides that are recognised by the DraIII restriction endonuclease. The presence of the GC bases in the 235–236 nt instead of CG bases is reported as a specific site for restriction cleavage by DraIII, and can be used when *Smallanthus sonchifolius* confirmation is needed, because none of the other ITS of the *Smallanthus* species has the DraIII recognition site in that location (Žiarovská *et al.* 2013). These findings were the basis for using the PCR-RFLP based approach to detect yacon in real herbal tea mixtures, as is presented here. PCR-RFLP was reported by Espiñeira *et al.* (2010) as an alternative to sequencing, and it has been widely used in many previous analyses to confirm the identity of a particular PCR product (Espiñeira *et al.* 2008).

The results of this study show the reliability of PCR and restriction cleavage of the ITS as a combined approach to confirm the presence of yacon in herbal tea mixtures. The advantages of the ITS regions made them a preferred choice in the past, as the ITS sequences were used as genetic markers for various species (Gao *et al.* 2010; Sun *et al.* 2011; Lin *et al.* 2012; Ganopoulos *et al.* 2012; Liu *et al.* 2012).

Slanc *et al.* (2006) used ITS for the identification of four herbal drugs in tea mixtures. The authors used an analysis and real-time PCR based approach for the identification of *Valerianae radix*, *Lupuli strobuli*, *Melissae folium*, and *Menthae piperitae folium*.

DART/TOF-MS method. The samples of tea mixtures and pure yacon tea were analysed by the DART/TOF-MS method and statistically processed by the principal component analysis (PCA) based on the fingerprints of the complete TIC mass spectra. First, the method was validated. The samples were measured in both a positive and a negative ion mode. As a result, only the negative mode was used, which provided a richer spectrum. The autosampler velocity and the location of the sampling rods in the autosampler were set according to Rajchl *et al.* (2013). The optimal conditions for the analysis of the samples were: negative mode, ionisation temperature of 450°C, and methanol as an extraction solution (Mangoni *et al.* 2011). The examples of MS spectra of pure yacon tea, herbal mixture 3, and herbal mixture 3 with yacon tea added are shown in Figure 4. It is evident from Figure 4 that there are significant differences in the MS spectra between the measured samples. The PCA analysis of herbal teas classified the samples of herbal teas into groups according to the first and second components (Figure 5). The PCA analysis shows sufficient separation of the herbal teas with and without yacon leaves. Sample 4 (pure yacon tea) is located completely apart from the other samples in the PCA plot. It is evident from the results that the DART/TOF-MS technique has a great potential for the evaluation of the quality and authenticity of herbal tea mixtures.

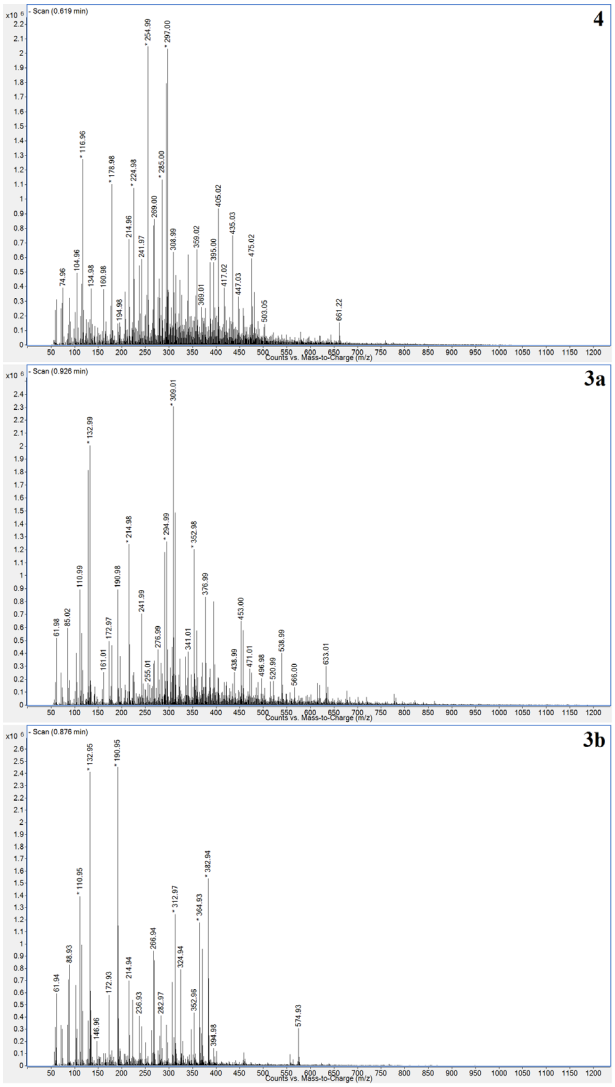

Figure 4. Total ion current (TIC) spectrum plots obtained from samples of pure yacon tea (4), herbal mixture 3 (3a), and herbal mixture 3 with the addition of yacon (3b)

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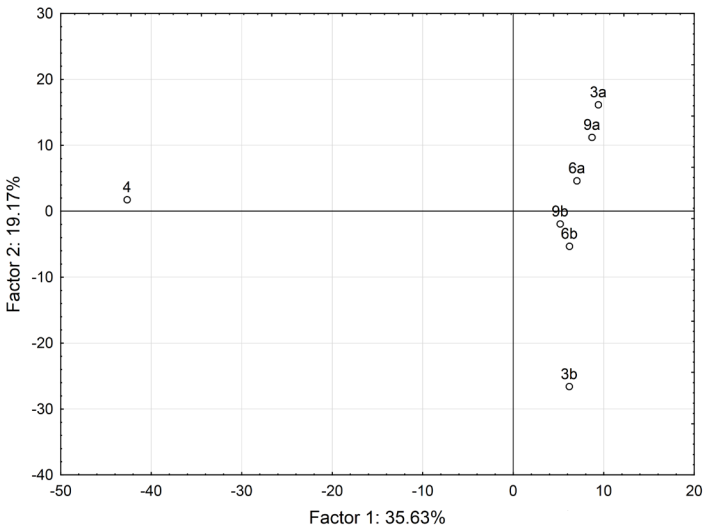

Figure 5. Principal component analysis (PCA) of the mass spectra of the measured samples

Both methods (ITS and DART) can be used successfully for the detection and identification of plant species in food products, for example in tea mixtures containing yacon.

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