



## Original Research Article

Tracing *Styphnolobium japonicum* (syn: *Sophora japonica*) as a potential adulterant of ginkgo-containing foods by real-time PCRLiliana Grazina<sup>a</sup>, Joana S. Amaral<sup>b</sup>, Joana Costa<sup>a</sup>, Isabel Mafra<sup>a,\*</sup><sup>a</sup> REQUIMTE-LAQV, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal<sup>b</sup> Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Sta. Apolónia, 5301-857, Bragança, Portugal

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## ABSTRACT

The rising demand for ginkgo-containing products and their high economic value make them desirable targets for adulteration, particularly by the partial substitution with other plant species. *Styphnolobium japonicum* (plant rich in flavonol glycosides) is known as a potential adulterant of ginkgo-based foods. Therefore, this work aimed at developing a species-specific real-time polymerase chain reaction (qPCR) method for the identification/quantification of *S. japonicum* as an adulterant of ginkgo-containing products. The method used the EvaGreen dye, targeting the internal transcribed spacer 2 (ITS2) region of *S. japonicum*, providing acceptable performance parameters and a sensitivity down to 0.02 pg of DNA. Moreover, a qPCR assay was established using binary mixtures of *S. japonicum* in *G. biloba*, covering the dynamic range of 50–0.05% (w/w) of added adulterant. After trueness evaluation with blind samples, the approach was applied to 21 commercial herbal infusions, from which one was positive to *S. japonicum*, but below the limit of quantification (0.05 %), suggesting its inadvertent contamination rather than adulteration. To the best of our knowledge, for the first time, a specific method was proposed to quantify potential adulterations of *G. biloba* products with *S. japonicum*, providing an accurate and cost-effective tool to authenticate ginkgo-containing herbal foods.

## 1. Introduction

*Ginkgo biloba* L. (known as ginkgo), a millenary Chinese tree also known as a living fossil, is one of the most popular medicinal plants for its beneficial activity in brain function. Currently, ginkgo is reported as one of the most popular botanicals used in plant food supplements (Garcia-Alvarez et al., 2014; Clarke et al., 2015), being legally considered as foods under the Directive, 2002/46/EC and the Dietary Supplement Health and Education Act (DSHEA) (FDA, 2019). Therefore, it is widely present as an ingredient in several food products, such herbal infusions and plant food supplements. Besides, it has been used by the pharmaceutical industry in the form of standardised extracts with  $\geq 24$  % of flavonol glycosides and  $\geq 6$  % of terpene lactones (Ma et al., 2016). The consumption of these products has been increasing over the last decade in European countries and USA, which is probably related with the rising number of elderly suffering from cognitive impairment and mild dementia due to the average life expectancy increase, as well as to a

growing number of consumers that want to improve general brain health and memory. The well-established biological effects of ginkgo in improving age-associated cognitive impairment and minor circulatory disorders are attributed to the presence of pharmacologically active compounds, particularly the flavonol glycosides and the terpene lactones (Booker et al., 2016; EMA, 2015; Gafner, 2018; WHO, 2004; Wohlmuth et al., 2014).

The rising sales of ginkgo herbal products and ever-growing demand for plant material resulted on its worldwide price increase, thus turning this species a desirable target of economically motivated adulteration. Up until now, several studies have shown ample evidences of fraud in *G. biloba* supply chains (Avula et al., 2015; Collins et al., 2020). The most frequently reported concern either the addition of pure flavonols/-flavonol glycosides or the total/partial replacement of ginkgo by other plant species rich in those compounds, but of lower economic value. Among these, *Styphnolobium japonicum* (L.) Schott (syn: *Sophora japonica* L.) has been referred as the most frequently used as *G. biloba* substitute

**Abbreviations:** PCR, Polymerase chain reaction; qPCR, quantitative real-time polymerase chain reaction; ITS, Internal transcribed spacer; HPLC, High performance liquid chromatography; RPA, recombinase polymerase amplification; *matK*, maturase K; *rbcL*, ribulose-bisphosphate carboxylase gene; LOD, limit of detection; LOQ, limit of quantification; Cq, cycle of quantification.

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**Table 1**Data of primers used, targeting the ITS2 region of *S. japonicum* and a conserved eukaryotic region.

Species	Target	Primer	Sequence (5' → 3')	Length	Reference
<i>S. japonicum</i>	ITS2	Styj3-F	CACGATGGATGGTGGCTGAG	110 bp	This work
		Styj3-R	TCTCGTTATGGGGCGATCGTA		
		18SRG-F	CTGCCCTATCAACTTCGATGGTA	113 bp	
18SRG-R	TTGGATGTGGTAGCCGTTTCTCA				
Eukaryotes	Nuclear 18S rRNA	EG-F	TCGATGGTAGGATAGTGGCCTACT	109 bp	Villa et al. (2017)
		EG-R	TGCTGCCTCTTGGATGTGGTA		

in food supplements (Avula et al., 2015; Budec et al., 2019; Gafner, 2018; Govindaraghavan, 2018). *S. japonicum* is used in traditional Chinese medicine for its haemostatic properties and has been described as a commercial source of rutin owing to its high levels in flowers and buds (Kite et al., 2009). This species is particularly suited to carry out such adulteration because its fruits are rich in flavonoid glycosides that tend to overlap with those of ginkgo (Govindaraghavan, 2018).

Currently, methods described in ginkgo quality control monographs rely mainly on the determinations of the ratios of flavonol aglycones (quercetin, kaempferol, and isorhamnetin) by high performance liquid chromatography (HPLC) after an acidic hydrolysis step and subsequent calculation of flavonol glycosides (Ma et al., 2016). However, such approaches can be inadequate when applied to formulations that include mixtures of plant species and can also fail to detect the addition of similar extracts from other plant sources. Alternatively, DNA-based techniques are highly suitable tools for species identification, with different works demonstrating their usefulness in the authentication of herbal infusions and plant food supplements (Costa et al., 2016; Grazina et al., 2020a, b). These methodologies take advantage of the high specificity of the DNA molecules, which exist in all plant cells and are independent from environmental, physiologic and storage conditions, allowing to overcome some limitations of the chemical methods (Costa et al., 2015; Ganie et al., 2015; Soares et al., 2015; Techen et al., 2014). So far, different DNA-based approaches have been reported aiming the specific identification of *G. biloba* in herbal products, namely DNA barcoding applied to dietary supplements (Little, 2014; Pawar et al., 2017), next generation sequencing applied to products containing ginkgo extracts or plant material plus extract (Ivanova et al., 2016) and real-time polymerase chain reaction (PCR) for the specific detection of *G. biloba* in natural health products (Dhivya et al., 2019) and herbal infusions (Grazina et al., 2020b). However, the detection of *S. japonicum* as ginkgo's adulterant has been reported only in one previous work. Liu et al. (2018) reported a recombinase polymerase amplification (RPA) approach relying on the use of species-specific primers and a probe with high specificity for the detection of both *G. biloba* and *S. japonicum* in herbal products. Nevertheless, the method does not allow for quantification and cross-reactivity testing with several other plant species was rather limited. Thus, in this work, a simple quantitative real-time PCR (qPCR) method using the EvaGreen dye was proposed and validated to detect and quantify *S. japonicum* as an adulterant in ginkgo-containing foods. The method was further applied to several ginkgo-based herbal infusions broadly available in the global market to assess any eventual substitution of *G. biloba* by *S. japonicum*.

## 2. Materials and methods

### 2.1. Plant species, commercial samples and model mixtures

*Styphnolobium japonicum* leaves and voucher seeds were kindly provided by the Botanical Garden of University of Trás-os-Montes e Alto Douro (UTAD) (Vila Real, Portugal) and University of Arizona Herbarium/Boyce Thompson Arboretum (USDA-Grin, Tucson, AZ, USA), respectively, while *G. biloba* leaves were from the Botanical Garden of University of Porto (Porto, Portugal). A total of 21 samples of ginkgo herbal infusions were acquired at the local market, specialised herbal

stores and e-commerce.

Seeds were grinded with a mortar, while the leaves and herbal infusions were grinded in a laboratory mill Grindomix GM200 (Retsch, Haan, Germany). Biological material of several plant species including medicinal and aromatic plants, spices and fruits were also used to for cross-reactivity testing (Table S1).

For the method development, model mixtures with 50 %, 10 %, 5 %, 1 %, 0.5 %, 0.1 %, 0.05 % and 0.01 % (w/w) of *S. japonicum* in *G. biloba* were prepared, using both species in the form of powdered leaves. Moreover, for trueness evaluation, four blind mixtures were also prepared (20.0 %, 8.0 %, 2.0 % and 0.2 % (w/w) of *S. japonicum* in *G. biloba*).

### 2.2. DNA extraction and quality assessment

DNA was extracted from 50 mg of powdered plant material using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with slight modifications, as described by Costa et al. (2016).

Yield and purity of the DNA extracts were evaluated by UV spectrophotometry, using a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT) with a Take3 micro-volume plate accessory and the nucleic acid quantification protocol for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc.). After quality assessment, all the extracts were kept at -20 °C until further analysis.

### 2.3. Oligonucleotide primers

*In silico* analysis was performed using the basic local alignment search (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) tools to verify the specificity of the target fragment and primers, respectively. The designed specific primers for *S. japonicum* (Styj3-F/Styj3-R) targeted the internal transcribed space 2 (ITS2) of nuclear ribosomal DNA (GenBank accession: HQ229005.1). Properties of the primers were checked using the OligoCalc software (<http://www.basic.northwestern.edu/biotools/oligocalc.html>) to guarantee the absence of primer hairpins and self-hybridisation. The presence of amplifiable DNA was confirmed using two universal eukaryotic primer pairs (18SRG-F/18SRG-R and EG-F/EG-R), targeting a conserved 18S rRNA nuclear region (Costa et al., 2013; Villa et al., 2017). The primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany) and are presented in Table 1.

### 2.4. End-point PCR

PCR amplification was carried out using 2 µL (20 ng) of DNA extract in a total reaction volume of 25 µL containing buffer (67 mM Tris-HCl, pH 8.8, 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01 % Tween 20), 3 mM of MgCl<sub>2</sub>, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience GmbH, Ulm, Germany), 200 nM of each primer (Styj3-F/Styj3-R), and 200 µM of each dNTP (Grisp, Porto, Portugal). The reactions were performed in a MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following temperature program: initial denaturation

at 95 °C for 5 min; 40 cycles of amplification at 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s; final extension at 72 °C for 5 min.

PCR products were analysed by electrophoresis in a 1.5 % agarose gel containing 1× Gel Red (Biotium, Inc., Hayward, CA, USA) for staining and carried out in 1 × SGTB buffer (GRISP, Porto, Portugal) running 20–25 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was recorded using Image Lab software version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA).

### 2.5. Real-time PCR

The qPCR assays were carried out in a total reaction volume of 20 µL, containing 2 µL (20 ng) of DNA, 1× SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 300 nM of each primer (Styj3-F/Styj3-R) (Table 1). A fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) was used under the following conditions: 95 °C for 5 min; 45 cycles at 95 °C for 15 s and 63 °C for 45 s, with collection of fluorescence signal at the end of each cycle. For melting curve analysis, the PCR amplicons were denatured at 95 °C for 1 min and then annealed at 63 °C for 3 min to allow the correct annealing of the DNA duplexes. Afterwards, the temperature was increased from 63 °C to 95 °C with increments of 0.2 °C every 5 s, with acquisition of fluorescence signal at the end of each melting temperature. Data were processed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA). Each sample was analysed in, at least, four replicates in two independent runs.

## 3. Results and discussion

### 3.1. Selection target region and evaluation of the oligonucleotide's specificity

Several regions, including nuclear and plastid regions such as *matK*, *rbcl*, ITS1 and ITS2, have been previously proposed as plant barcodes (Grazina et al., 2020b; Hollingsworth et al., 2009, 2011). Among them, the nuclear ITS region has been described as a valuable DNA barcode for molecular authentication of plant species due to its high interspecific divergence and high discrimination power over plastid regions (Li et al., 2015). Moreover, the ITS2 region has already been suggested as a universal DNA barcode for the differentiation of plant species because it presents enough variability to distinguish even closely related species, being generally a region of easy amplification (Yao et al., 2010). Therefore, in this work, the nuclear ITS2 region was chosen to design specific primers for *S. japonicum* to allow the amplification of a short fragment of 110 bp (Styj3-F/Styj3-R). The optimised species-specific PCR reached a sensitivity down to 0.002 pg of *S. japonicum* DNA (supplementary Fig. S1).

The absence of sequence homology with other species was verified using the BLAST tool, while the primer specificity was checked with the Primer-BLAST tool. The primer specificity was further assayed using DNA extracts obtained from several plant species, including medicinal plants, fruits and spices, among others, confirming the absence of amplification (supplementary Table S1). Before cross-reactivity testing, the amplification capacity of DNA extracts was verified by PCR targeting a eukaryotic fragment, as described by Costa et al. (2013).

### 3.2. Real-time PCR

#### 3.2.1. Method development

After demonstrating the adequacy of the new primers for the specific identification of *S. japonicum* DNA, a qPCR method using EvaGreen dye was developed. Comparing with the oligonucleotide probes, the universal dyes offer a greater flexibility and reduced cost, allowing also to verify the reaction specificity by means of the dissociation (melt) curve analysis of the PCR products. EvaGreen is a new generation fluorescent

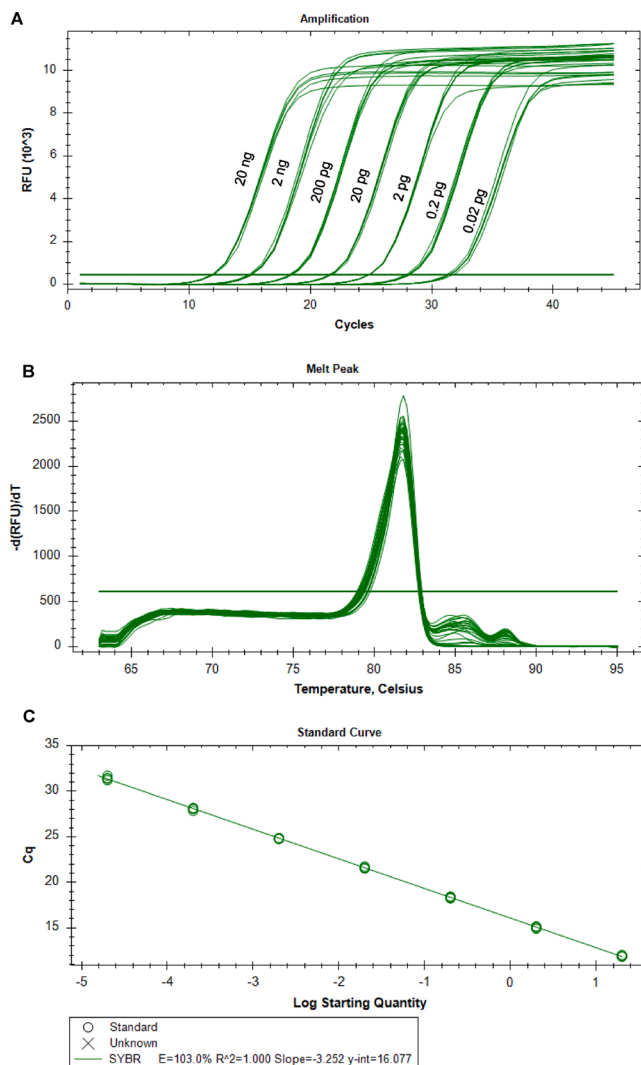
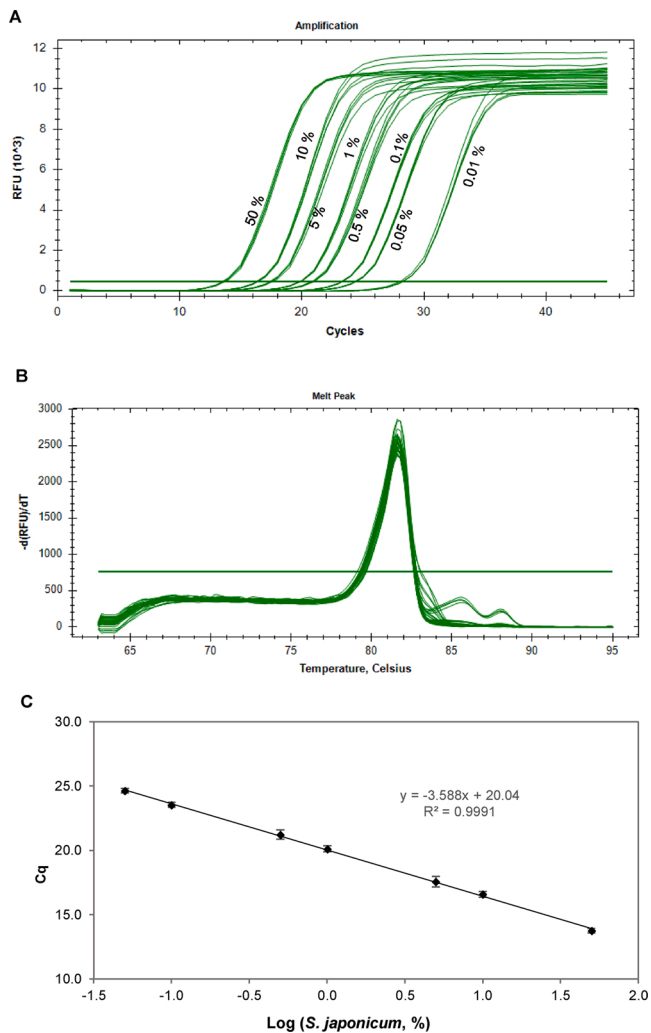


Fig. 1. Amplification (A), melting (B) and calibration (C) curves of a real-time PCR assay with EvaGreen® dye, targeting the ITS2 region of *S. japonicum*. The amplified extracts were obtained from 10-fold serially diluted *S. japonicum* DNA from 20 ng to 0.02 pg ( $n = 5$  replicates).

DNA-binding dye that offers several advantages over the widely used SYBR Green I dye. Enhanced fluorescence of dsDNA binding products, excellent stability and ability to be used at high concentration without inhibiting the PCR are among the superior features of the EvaGreen dye, which results in high reaction efficiencies (Eischeid, 2011). These unique properties allow for increased sensitivity and make EvaGreen dye particularly suited for qPCR.

For method development and validation, the acceptance criteria established for qPCR assays were carefully considered, namely the slope within -3.6 and -3.1, the PCR efficiency between 90–110 %, and the correlation coefficient ( $R^2$ ) >0.98 (Bustin et al., 2009; ENGL, 2015). The limit of detection (LOD) and the limit of quantification (LOQ) were established as the lowest amplified level for 95 % of the replicates and the lowest amplified level within the linear dynamic range of the calibration curve provided the relative repeatability standard deviation is ≤25 %, respectively. The linear dynamic range of the calibration curve should cover at least 4 orders of magnitude (Bustin et al., 2009; ENGL, 2015).

Before method development, additional cross-reactivity tests were performed by real-time PCR to confirm the specificity of the primers for *S. japonicum* identification as a potential adulterant of *G. biloba*. With this aim, a qPCR amplification was carried out using extracts containing



**Fig. 2.** Amplification (A), melting (B) and calibration (C) curves of a real-time PCR assay with EvaGreen® dye, targeting ITS2 region of *S. japonicum*, using the binary mixtures with 50 %, 10 %, 5 %, 1 %, 0.5 %, 0.1 %, 0.05 % and 0.01 % (w/w) of *S. japonicum* in *G. biloba* (mean values of 6 independent assays in triplicate,  $n = 18$ ).

20 ng of each of *G. biloba* and *S. japonicum* DNA (corresponding to the higher level of the calibration curve). The results show an unspecific amplification of ginkgo DNA (20 ng), but at much higher Cq (cycle of quantification) values ( $Cq = 32.21 \pm 0.09$ ) (supplementary Fig. S1) than the same amount of the target *S. japonicum* DNA ( $Cq = 12.12 \pm 0.04$ ). Therefore, a Cq value of 32.00 cycles was established as a threshold for specificity, allowing to avoid any false-positive.

For method development, a series of 10-fold serially diluted extracts were prepared covering 8 concentration levels, from 20 ng to 0.002 pg of *S. japonicum* DNA, whose amplification curves are shown in Fig. 1A. The respective melting curves show close profiles and melt peaks of  $81.7 \pm 0.1$  °C, which are characteristic of the formation of one single type of fragment and indicates the absence of unintended amplicons (Fig. 1B). The amplification of all replicates ( $n=10$ ), in two independent assays allowed establishing an absolute LOD of 0.02 pg ( $Cq = 31.43 \pm 0.20$ ) of *S. japonicum* DNA. The lowest tested level of 0.002 pg ( $Cq = 34.87 \pm 1.01$ ), though still amplifying all replicates, it could not be considered as LOD because the obtained Cq values were above the established threshold of specificity. The LOQ value was considered the same as the LOD since it was within the dynamic range of the calibration curve, having a relative repeatability standard deviation of 0.64 %, thus much below the acceptance criteria ( $\leq 25$  %) (Fig. 1C).

**Table 2**

Results of the trueness evaluation of the method based on the application of the developed quantitative PCR system to blind mixtures of *S. japonicum* in *G. biloba*.

Blind Mixtures	<i>Styphnolobium japonicum</i> (% w/w)		CV <sup>b</sup> (%)	Error <sup>c</sup> (%)
	Real value (%)	Estimated value <sup>a</sup> (%)		
SG_A	20.0	18.3 ± 2.7	14.5	-8.50
SG_B	8.0	6.80 ± 1.42	20.9	-15.0
SG_C	2.0	1.50 ± 0.18	11.9	-24.8
SG_D	0.2	0.19 ± 0.01	6.59	-3.74

<sup>a</sup> Mean value ± standard deviation (SD) ( $n = 6$ ) of two independent assays.

<sup>b</sup> Coefficient of variation (CV).

<sup>c</sup> Error = ((mean estimated value - real value)/real value) x 100.

The average real-time PCR performance parameters of several assays, namely the PCR efficiency ( $103.4 \pm 0.5$  %), the slope ( $-3.244 \pm 0.011$ ) and the  $R^2$  ( $0.999 \pm 0.001$ ), were all in accordance with the acceptance criteria, as evidenced in the example (Fig. 1C) (Bustin et al., 2009; ENGL, 2015).

Subsequently, aiming at proposing a quantitative model and establishing the relative sensitivity of the method, the binary mixtures containing 50 %, 10 %, 5 %, 1 %, 0.5 %, 0.1 %, 0.05 % and 0.01 % (w/w) of *S. japonicum* in *G. biloba* were amplified by real-time PCR (Fig. 2A). The respective melting curve analysis showed the presence of a single melting peak at  $81.7 \pm 0.1$  °C, which corroborates the suitability of the designed primers, ensuring the absence of primer dimmer (Fig. 2B). To construct the relative standard calibration curve, the obtained Cq values were plotted against the logarithm of the respective relative amounts of *S. japonicum* (Fig. 2C). The obtained calibration curve shows a high performance of the analytical approach since the mean values of PCR efficiency (90.0 %),  $R^2$  (0.999) and slope (-3.588) were all within the acceptance criteria for this type of assays (Bustin et al., 2009; ENGL, 2015). The relative LOD was down to 0.01 % (w/w), while a LOQ of 0.05 % was established considering the dynamic range of the respective curve, having a relative repeatability standard deviation of 0.72 %, which was much below the acceptance criteria ( $\leq 25$  %).

### 3.2.2. Trueness

For the evaluation of method trueness, four mixtures of *G. biloba* powdered leaves spiked with 20.0 %, 8.0 %, 2.0 % and 0.2 % (w/w) of *S. japonicum* were analysed as blind samples, aiming at evaluating the precision and accuracy of the approach (Bustin et al., 2009; ENGL, 2015). Table 2 shows the results of the estimated relative amounts of *S. japonicum* and the respective actual values. The precision, determined by the coefficients of variation (CV) values (relative standard deviation of results obtained under repeatability conditions), ranged between 6.59 % and 20.9 %, which demonstrates the good precision of the method ( $\leq 25$  %) over the considered dynamic range. The measured trueness was expressed as the bias or error between the estimated and the actual values varied from -3.7 % to -24.8 %, thus being within  $\pm 25$  % and indicating a good accuracy of the method (ENGL, 2015).

### 3.3. Analysis of ginkgo herbal infusions

The assessment of food authenticity is an issue of increasing concern for governmental bodies, industries and consumers. Herbal infusions are highly consumed and easily available products in the global market, with their formulation frequently including medicinal plants with specific therapeutic effects. Therefore, any adulteration by species substitution will compromise the integrity, and eventually the safety, of the final product, thus making the authenticity assessment of these products of major relevance. To detect possible adulteration with *S. japonicum*, 21 commercial ginkgo-containing herbal infusions were acquired at traditional, specialised and e-commerce stores. After ensuring the presence of amplifiable DNA, each sample extract was amplified by qualitative PCR assay with the new species-specific primers (Styj3-F/Styj3-R). The PCR

**Table 3**  
Results of commercial samples analysis by end-point PCR and qPCR, targeting the ITS2 region of *S. japonicum*.

Commercial samples	Label	End-point PCR <sup>c</sup>		qPCR		
		18SRG-F/18SRG-R	Styj3-F/Styj3-R	Cq (mean ± SD) <sup>a</sup>	Estimated <i>S. japonicum</i> (mean ± SD, %, w/w) <sup>b</sup>	
1	Ginkgo tea (leaves)	100 % ginkgo	+	–	ND	
2	Ginkgo tea (leaves)	100 % ginkgo	+	–	ND	
3	Ginkgo tea (leaves)	100 % ginkgo	+	–	ND	
4	Herbal infusion (sachets)	15 % ginkgo	+	–	ND	
5	Ginkgo infusion (sachets)	100 % ginkgo	+	+	>32.00	
6	Ginkgo infusion (sachets)	100 % ginkgo	+	+/-	>32.00	
7	Ginkgo tea (leaves)	100 % ginkgo	+	–	ND	
8	Ginkgo bio-infusion (leaves) (Agriculture non-EU)	100 % ginkgo	+	–	ND	
9	Ginkgo tea (leaves) (Agriculture non-EU)	100 % ginkgo	+	–	ND	
10	Tisana MC (Plant mixture)	15 % ginkgo	+	–	ND	
11	Tisana TB (Plant mixture)	15 % ginkgo	+	–	ND	
12	Ginkgo tea (sachets)	30 % ginkgo	+	+	30.50 ± 0.19	< 0.01 (LOD <sup>e</sup> )
13	Ginkgo tea (sachets)	100 % ginkgo	+	+	27.28 ± 0.06	< 0.05 (LOQ <sup>f</sup> )
14	Herbal tea (sachets)	Ginkgo (% not labelled)	+	–	ND	
15	Herbal tea (sachets)	Ginkgo (% not labelled)	+	–	ND	
16	Herbal tea (sachets)	Ginkgo (% not labelled)	+	–	ND	
17	Herbal tea (sachets) (Agriculture non-EU)	15 % ginkgo	+	–	ND	
18	Ginkgo tea (leaves)	100 % ginkgo	+	–	ND	
19	Ginkgo tea (leaves)	100 % ginkgo	+	+	29.05 ± 0.16	< 0.01 (LOD)
20	Ginkgo tea (sachets)	100 % ginkgo	+	+/-	>32.00	
21	Ginkgo tea (leaves)	100 % ginkgo	+	+/-	>32.00	

<sup>a</sup>Mean cycle of quantification (Cq) values ± standard deviation (SD) (n = 4). <sup>b</sup>Mean percentage (%) values ± SD (n = 4). <sup>c</sup>(+) Positive amplification; (–) Negative amplification; +/- Doubtful amplification. <sup>d</sup>ND, not determined. <sup>e</sup>LOD, limit of detection. <sup>f</sup>LOQ, limit of quantification.

results showed that 4 out of the 21 commercial samples presented clear positive amplification for *S. japonicum*, while other 3 presented faint bands. To confirm these results and estimate the content of a possible adulterant, the developed qPCR system was applied to the mentioned 7 samples. As it can be observed in Table 3, samples #5, #6, #20 and #21 should be considered as negative since they presented Cq values above the threshold level (32 cycles), which agrees with the qualitative PCR results. Regarding samples #12 and #19, although having Cq values below 32, according to the validated calibration model of Fig. 2C, the estimated *S. japonicum* was below the LOD of 0.01 %, thus being considered negative. For the remaining sample #13 the obtained Cq value was 27.28, corresponding to an estimated value of 0.011 ± 0.000 %, which is below the LOQ. Therefore, such trace amount of *S. japonicum* suggest inadvertent contamination rather than a potential adulteration.

#### 4. Conclusions

In this work, a qPCR approach using the fluorescent EvaGreen dye was developed aiming at the specific detection and quantification of *S. japonicum* as a potential adulterant of *G. biloba*. The approach targeted the ITS2 region of *S. japonicum* that proved to be a specific marker for this species. The ITS2 marker allowed reliably detecting down to 0.02 pg of *S. japonicum* DNA using both qualitative PCR and qPCR. To develop a quantitative approach, model mixtures of powdered leaves of *S. japonicum* in *G. biloba* were prepared and analysed by qPCR, which covered a range from 50 % to 0.01 % (w/w) of added adulterant. The obtained calibration model provided acceptable performance parameters of PCR efficiency, slope and correlation coefficient, having a LOD and a LOQ of 0.01 % and 0.05 %, respectively. The method was successfully validated using four blind mixtures of known composition, showing adequate precision and accuracy. The application of the proposed approach to commercial ginkgo-based herbal infusions showed that out of 21 samples, 7 were suspected as having the *S. japonicum* DNA by qualitative PCR. However, its presence was confirmed in only one sample by qPCR, at a trace levels below the LOQ, suggestion a possible inadvertent contamination, rather than an adulteration.

In summary, the present work proposes a novel, reliable, highly sensitive and specific tool that could be useful to detect potential adulterations of ginkgo-containing herbal products by the control

laboratories and regulatory authorities, ensuring their authenticity and safety.

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#### CRedit authorship contribution statement

**Liliana Grazina:** Methodology, Formal analysis, Investigation, Writing - original draft. **Joana S. Amaral:** Conceptualization, Supervision, Writing - review & editing. **Joana Costa:** Formal analysis, Investigation, Writing - review & editing. **Isabel Mafra:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

#### Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

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