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A Selective Ion HPLC-APCI-MS Method for the Quantification of Pentacyclic Triterpenes in an Anxiolytic Botanical Dietary Supplement for the Animal Health Market

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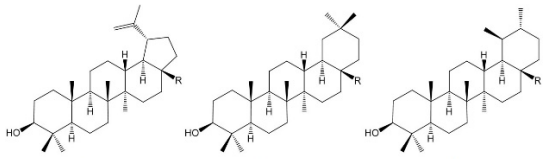
A new anti-anxiety dietary supplement was developed for the animal health market, by combining 2 triterpene containing botanicals, *Souroubea sympetala* (Marcgraviaceae) with *Platanus occidentalis* (Platanaceae) A validated method for a quality control of the botanical blend was achieved using HPLC-APCI-MS. The method resulted in the detection and quantitative determination of betulinic acid (**1**), and ursolic acid (**2**) in *P. occidentalis* and **1**, **2**, lupeol (**3**), β-amyrin (**4**) and α-amyrin (**5**) in *S. sympetala* and the finished product Zentrol™. Detections were at low ng on column for **1** and **2** and in low µg range for **3**, **4** and **5** using calibration curves within 10-100 ng ($R^2 > 0.993$). Recovery of spiked samples for all the recoveries observed were > 94%. Inter-day and intra-day variations were 0.8-3.5% and 5-10.4%, respectively. These results indicate the suitability of the developed analytical method to detect and quantify triterpenes of raw materials used in the manufacture of natural health products.

Keywords: Pentacyclic triterpenes, Quality control, *Souroubea*, *Platanus*, HPLC-APCI-MS.

The diverse bioactivities and low toxicity of triterpene rich plant extracts are a good choice for phytopharmaceutical products [1, 2]. Specially, pentacyclic triterpenes, are well known for their anxiolytic, anti-depressant, antibacterial, anti-inflammatory, anti-HIV and anti-cancer effects [3, 4]. Our collaborative research group in Canada and Costa Rica, has focussed on the anxiolytic properties of several plants containing pentacyclic triterpenes. The genus *Souroubea* (Marcgraviaceae) includes 19 species distributed over much of the neotropics but absent from the Antilles [4]. *Souroubea guianensis* Aublet var cylindrica Wittm. was used to treat mood and sleep disorders [5,6]. In our studies, extracts of leaf and stem of two Central American species, *Souroubea gilgii* Gilg and *Souroubea sympetala* V.A. Richt were found to have significant activity in rat behavioral models of anxiety such as the elevated plus maze [7]. Both species have a similar phytochemical profile and anxiolytic activity guided fractionation yielded triterpene rich fractions containing betulinic acid (**1**), ursolic acid (**2**), lupeol (**3**), β-amyrin(**4**) and α-amyrin (**5**) (Figure 1) [8]. Betulinic acid was found to be the main active principle [8]. Amyrins were also reported to have anxiolytic effect *in vivo* [9].

Further field work in Ontario, Canada identified Sycamore (*Platanus occidentalis* L., Platanaceae) a native North American tree, as an excellent source of 1 from the bark. A proprietary blend of *Souroubea* spp. with *Platanus* spp. herbal ingredients (Zentrol™) was developed which was found to be more efficacious in animal trials and more cost effective than either botanical alone [10]. For any new botanical dietary supplement, quality control of active



Terpene Family	Triterpene	R	Neutral Mass	Designates Compound Number
Lupane	Betulinic acid	COOH	456.36	1
Ursane	Ursolic acid	COOH	456.36	2
Lupane	Lupeol	CH3	427.73	3
Oleanane	β-amyrin	CH3	427.73	4
Ursane	α-amyrin	CH3	427.73	5

Figure 1: Pentacyclic triterpenes identified *in Souroubea* spp and *Plantanus* spp.

principles is important to be able to compare the eventual product used with clinically tested material. As well, quality control is essential to select the best genotypes for use and preparation of formulated material. A pilot study described the chromatographic separation and detection of four triterpenes (Figure 1) in *Souroubea* spp. [11] but was not applied to *Platanus* spp. or validated for quantitative analytical use. In this report, our first objective was the development and validation of an HPLC-APCI-MS method for analysing and fully quantifying 5 pentacyclic triterpenes in *S. sympetala*, *P. occidentalis* raw materials and finished blended product. The second objective was to determine amounts of the 5 triterpenes in potential source materials including *S. sympetala*., *S gilgii, P. occidentalis, Platanus x acerifolia* (Aiton) Willd. and *P. mexicana* Moric.

Table 1: Chromatographic, spectrometric and validation data of pentacyclic triterpenes identified from *S. sympetala* by the developed analytical methodology. Limits of detection (LOD, 3:1 signal to noise) and limits of quantification (LOQ, 10:1 signal to noise), of each marker on column (6 x standard deviation) in leaf and stem of *S. sympetala.* R^2 value of linear regressions >0.99. Mean (SEM) are presented for % Intraday variation (n=10), % Interday variation (n=10) and % recovery (n=3).

Triterpene	RT (min)	Ion detected	LOD (ng)	LOQ (ng)	% Intraday variation	% Interday variation	Linearity (R^2)	Range (µg/ml)	% recovery[a]	
									Souroubea	*Plantanus*
1	9.7	455.3, $[M\text{-}H^+]^-$	0.01	0.03	1.55 (0.4)	4.96 (0.7)	0.998	0.2-25.0	102.3 (2.1)	95.0 (3.0)
2*	9.8	455.3, $[M\text{-}H^+]^-$	6.6	21.9	3.46 (0.5)	5.66 (0.8)	0.997	0.2-10.0	95.1 (2.3)	91.0 (2.2)
3	13	425.4, $[M+H\text{-}2H]^+$	610	2030	2.71 (0.9)	10.34 (1.4)	0.993	1.0-100.0	96.5 (1.7)	NA
4	14.1	425.4, $[M+H\text{-}2H]^+$	363	1210	2.16 (0.2)	5.11 (0.7)	0.998	1.0-100.0	94.6 (1.5)	NA
5	14.2	425.4, $[M+H\text{-}2H]^+$	300	998	0.79 (0.2)	5.04 (1.0)	0.998	1.0-100.0	99.8 (1.0)	NA

*Quantified on the basis of peak height. [a] Determined from the slope of regression analysis of recovered quantities with amount spiked.

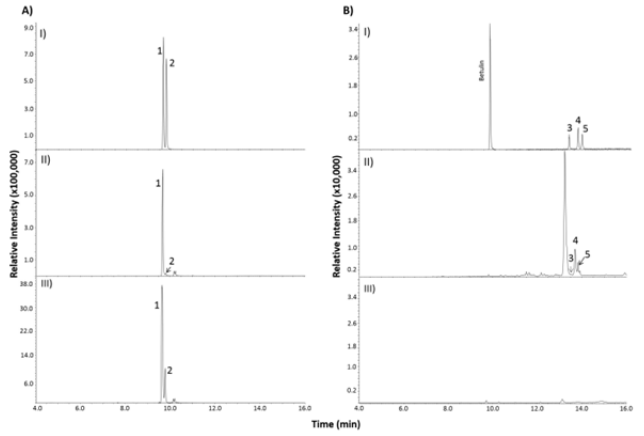


Figure 2: Chromatographic separation of triterpenes, betulinic acid (**1**), ursolic acid (**2**), lupeol (**3**), β-amyrin (**4**) and α-amyrin (**5**). A) HPLC-APCI negative Total ion chromatogram and B) HPLC-APCI positive ion chromatogram. I): standard compounds mixture, II) *S. sympetala*, III) *P. occidentalis*.

Figure 1 shows the neutral masses of the target compounds identified from raw materials of *Souroubea* spp, and *Platanus* spp. Compounds **1** and **2** produced two peaks at m/z 455.3 eluting at 9.7 and 9.8 min in negative ionization (Figure 2) and 3, 4, 5 produced three peaks at m/z 425.4 in positive ionization eluting at 13, 14.1 and 14.2 min respectively while betulin eluted at 10.1 min (Figure 2B). Since betulin was detected only at trace levels below detection limits in the study materials it was not included in method validation. The developed method enabled us to identify and quantify the markers in plant extracts with a linearity (R2>0.997) for all of the compounds within a range of 0.2-25.0 ng for betulinic acid on column, 0.2-10.0 ng of ursolic acid on column, and 1.0-100.0 ng on column for 3, 4 and 5 (Table 2). It is notable that proper integration is very important for authentic quantitation particularly for 1 and 2 with approximately 10% signal overlap and the presence of a semi co-eluting peak with 5. The appearance of additional signals with selected ion monitoring indicates the presence of analogues of amyrins. The sensitivity of the method was assessed by determination of limits of detection and quantification of the compounds under current extraction protocol (Table 1) The LOD and LOQ were much better (pg amounts on column) for **1** and **2** than other compounds 3-5. When recovery analyses of spiked samples were performed for the five triterpenes to determine accuracy, the linear regression of recovered amount versus the spiked were obtained (R^2 >0.99) and all the recoveries observed were > 94% (Table 1). Based on the results observed for the recoveries, the extraction described in the sample preparation is recommended for targeted analysis. However, in a previous study [12], results by supercritical extraction were similar, and this method could be substituted if desired and the equipment is available. Percentage recovery of 102 % for betulinic acid can be explained by the 1.55 % CV observed during intraday variation study. These results reveal that the method is highly reproducible. The precision of the method (Table 2), assessed by calculating the coefficient of variation between trials (intraday variation) did not exceed 3.5%. As expected, the interday variation was somewhat higher. It was approximately 5% for all compounds except for 3 (10%). In addition to the soxhlet method here, we previously tested supercritical extraction (SCE), assisted solvent extraction ASE and alcohol extraction [12]. Betulinic acid is difficult to fully extract and only SCE gave comparable yields of to the soxhlet method. It can be substituted in the analytical procedure, but it does require a considerable investment in equipment.

As phytochemical variations are quite common intra/inter species from the same family, we applied the validated method to a diversity of biological samples collected in different environments and representing typical botanical raw material for potential use in a natural health product. These included a number of S. *sympetala, S, gilgii, P. occidentalis, P. acerifolia* and *P. mexicanus* collections from North and Central America. During analysis all marker compounds were successfully resolved and quantified from these materials (Table 2). Based on the quantitative results obtained, all the selected triterpenes are present in detectable levels in both plant parts of *S. sympetala* and *S. gilgii*. There was a significant variation in the content of this triterpenes according to plant part. Betulinic acid is manly present in the stems. On the other hand, leaf extracts had higher content of α-amyrin and β-amyrin. There was considerable variation in content of the five triterpenes analysed in the samples evaluated. Betulinic acid content ranged from 0.12 to 7.2 mg/g in stem, lupeol and amyrins can vary up to 20 folds in leaves. For the preparation of the animal health product, we decided to use *S. sympetala* plant material from a plantation maintained by the Universidad Nacional, which had a mid level of triterpenes, but plants were produced in a uniform farm environment to reduce variation.

It is important to mention that *S. sympetala* and *S. gilgii* grow in the same locations and show the presence of the five triterpenes evaluated in the method in similar amounts. Both species are comparable in anxiolytic activity and show minor difference in phytochemistry and could be used as a mixed species product as is the case for Hawthorn for which a mixture of *Craetagus monogyna* Jacq and *Craetagus laevigata* (Poir DC) is used [13]. Among the three species of *Plantanus* collected in 27 sites across North America, betulinic acid is the dominant tritepene accompanied with ursolic acid. Amyrins and lupeol were not detected (Table 2). Betulinic acid concentrations varied from 5.7 to 22.1 mg/g in dried bark and ursolic acid concentration ranged from 0 to 3.36 mg/g. Overall, it can be observed that the *P.x acerifolia* exhibited the highest content of betulinic acid, while *P. occidentalis* had the lowest betulinic acid concentration among the three species. This study is the first comparative phytochemical analysis of betulinic acid and usrolic acid in the *Platanu*s genus. Due to the diverse biological effects of betulinic acid (anxiolyic, anti-cancer and anti-viral effects) [1,2], the findings of this study contribute to identifying Platanus sources with the highest content of bioactive ingredient.

Table 2: Quantification (mg of compound/g of dried plant) of five triterpenes in *Souroubea and Platanus* spp. Mean (Sem) of concentrations are presented.

Code	Location	Year	Plant organ	Sample size	Concentration in plant (mg/g)				
					1	2	3	4	5
SS-01	Barra Colorado, Costa Rica	2009	Stem	3	2.50 (0.02)	0.02 (0.00)	1.36 (0.03)	1.41 (0.03)	1.28 (0.02)
SS-02	Barra Colorado, Costa Rica	2009	Stem	3	2.91 (0.09)	0.06 (0.00)	1.65 (0.02)	1.37 (0.04)	1.75 (0.02)
SS-03	Sarapiqui, Costa Rica	2009	Stem	3	0.82 (0.01)	0.03 (0.00)	0.19 (0.00)	0.24 (0.01)	0.11 (0.00)
SS-04	Sarapiqui, Costa Rica	2009	Stem	3	2.61 (0.04)	0.27 (0.01)	0.81 (0.02)	0.93 (0.03)	0.19 (0.01)
SS-05	Sarapiqui, Costa Rica	2009	Stem	3	2.18 (0.03)	0.02 (0.00)	0.48 (0.01)	0.58 (0.04)	0.70 (0.01)
SS-06	Sarapiqui, Costa Rica	2009	Stem	3	0.12 (0.01)	0.01 (0.00)	0.04 (0.00)	0.03 (0.00)	0.01 (0.00)
SS-07	Sarapiqui, Costa Rica	2009	Stem	3	1.43 (0.00)	0.03 (0.00)	1.18 (0.03)	2.83 (0.09)	0.13 (0.01)
SS-08	Sarapiqui, Costa Rica	2009	Leaf	3	0.11 (0.00)	0.18 (0.01)	0.74 (0.05)	4.71 (0.06)	7.57 (0.09)
SS-09	Sarapiqui, Costa Rica	2009	Leaf	3	0.02 (0.00)	0.11 (0.00)	1.85 (0.07)	5.83 (0.17)	5.31 (0.17)
SS-10	Sarapiqui, Costa Rica	2009	Leaf	3	*	0.05 (0.00)	0.58 (0.01)	2.86 (0.07)	0.47 (0.02)
SS-11	Sarapiqui, Costa Rica	2009	Leaf	3	0.04 (0.00)	0.37 (0.02)	2.57 (0.09)	5.54 (0.09)	8.25 (0.07)
SS-12	Sarapiqui, Costa Rica	2011	Leaf	3	0.03 (0.00)	0.37 (0.01)	3.88 (0.17)	10.14 (0.45)	10.94 (0.6)
SS-13	Tirimbina, Costa Rica	2009	Stem	3	0.83 (0.01)	0.06 (0.00)	0.09 (0.00)	0.14 (0.01)	0.07 (0.00)
SS-14	Tortuguero, Costa Rica	2009	Stem	3	7.22 (0.24)	0.10 (0.00)	1.25 (0.02)	0.79 (0.01)	0.07 (0.00)
SS-15	Tortuguero, Costa Rica	2009	Stem	3	3.70 (0.12)	0.10 (0.00)	0.74 (0.02)	0.97 (0.03)	1.54 (0.04)
SS-16	Tortuguero, Costa Rica	2009	Stem	3	4.92 (0.14)	0.05 (0.00)	0.30 (0.02)	0.45 (0.03)	0.06 (0.00)
SS-17	Tortuguero, Costa Rica	2009	Leaf	3	#	#	0.71 (0.02)	5.12 (0.09)	7.00 (0.21)
SS-18	Tortuguero, Costa Rica	2009	Leaf	3	0.01 (0.00)	0.09 (0.00)	0.18 (0.00)	2.17 (0.02)	0.61 (0.01)
SS-19	Tortuguero, Costa Rica	2009	Leaf	3	0.01 (0.00)	0.45 (0.01)	1.66 (0.06)	6.63 (0.07)	8.12 (0.06)
SS-20	Tortuguero, Costa Rica	2009	Leaf	3	0.00 (0.00)	0.25 (0.01)	1.15 (0.04)	3.18 (0.02)	1.15 (0.04)
SS-21	Tortuguero, Costa Rica	2009	Leaf	3	0.00 (0.00)	0.09 (0.00)	0.66 (0.01)	3.38 (0.04)	4.20 (0.10)
SG-1	Cano Palma, Costa Rica	2010	Leaf	3	0.21 (0.12)	0.02 (0.01)	0.51 (0.02)	3.9 (0.08)	0.48 (0.03)
SG-2	Cano Palma, Costa Rica	2010	Stem	3	1.26 (0.73)	0.02 (0.01)	0.16 (0.00)	0.2 (0.01)	0.23 (0.01)
SG-3	Cano Palma, Costa Rica	2009	stem	3	4.38 (2.53)	0.05 (0.03)	0.83 (0.01)	0.94 (0.01)	0.53 (0.02)
SG-4	Horquetas, Costa Rica	2010	Stem	3	0.58 (0.33)	0.03 (0.02)	0.28 (0.00)	0.52 (0.01)	0.12 (0.00)
SG-5	Horquetas, Costa Rica	2011	Leaf	3	0.05 (0.03)	0.3 (0.18)	0.34 (0.01)	2.37 (0.06)	0.92 (0.01)
SG-6	Sarapiqui, Costa Rica	2010	stem	3	0.85 (0.49)	0.08 (0.05)	0.1 (0.00)	0.15 (0.01)	0.06 (0.00)
SG-7	Sarapiqui, Costa Rica	2009	Stem	3	0.7 (0.4)	0.04 (0.02)	0.36 (0.01)	0.95 (0.03)	0.02 (0.00)
SG-8	Sarapiqui, Costa Rica	2011	Stem	3	1.42 (0.82)	0.03 (0.02)	0.38 (0.01)	0.5 (0.01)	0.19 (0.00)
SG-9	Sarapiqui, Costa Rica	2011	leaf	3	0.18 (0.1)	0.34 (0.19)	1.05 (0.03)	4.74 (0.04)	11.95 (0.12)
SG-10	Sarapiqui, Costa Rica	2011	leaf	3	0.21 (0.12)	1.56 (0.9)	1.02 (0.03)	6.15 (0.08)	7.87 (0.15)
SG-11	Tirimbina, Costa Rica	2009	Leaf	3	0.09 (0.05)	0.01 (0.01)	0.96 (0.04)	4.07 (0.05)	3.45 (0.16)
SG-12	Tirimbina, Costa Rica	2011	Stem	3	1.15 (0.66)	0.06 (0.03)	0.08 (0.00)	0.1 (0.00)	0.13 (0.00)
SG-13	Tirimbina, Costa Rica	2011	Leaf	3	0.01 (0.1)	0.01 (0.01)	0.88 (0.05)	3.57 (0.07)	3.38 (0.06)
SG-14	Tortuguero, Costa Rica	2009	Stem	3	3.09 (1.78)	*	0.26 (0.01)	0.42 (0.01)	0.02 (0.00)
SG-15	Tortuguero, Costa Rica	2009	stem	3	1.13 (0.65)	0.04 (0.02)	0.52 (0.01)	0.44 (0.00)	0.49 (0.00)
SG-16	Tortuguero, Costa Rica	2010	Stem	3	2.04 (1.18)	0.01 (0)	0.73 (0.01)	0.75 (0.01)	0.8 (0.02)
SG-17	Tortuguero, Costa Rica	2010	stem	3	0.84 (0.49)	0.03 (0.02)	0.09 (0.00)	0.09 (0.01)	0.02 (0.00)
SG-18	Tortuguero, Costa Rica	2011	leaf	3	0.74 (0.43)	0.03 (0.02)	0.66 (0.01)	1.14 (0.02)	0.14 (0.01)
SG-19	Tortuguero, Costa Rica	2010	Leaf	3	0.1 (0.06)	0.02 (0.01)	0.54 (0.01)	3.8 (0.06)	2.27 (0.01)
SG-20	Tortuguero, Costa Rica	2010	Leaf	3	0.01 (0)	0.19 (0.11)	0.26 (0.00)	2.01 (0.03)	2.39 (0.06)
SG-21	Tortuguero, Costa Rica	2009	Leaf	3	0.2 (0.12)	0.7 (0.41)	0.81 (0.06)	4.92(0.36)	14.49 (0.56)
SG-22	Tortuguero, Costa Rica	2008	Leaf	3	1.25 (0.72)	0.03 (0.02)	1.71 (0.08)	6.66 (0.15)	4.75 (0.09)
PA-01	Essex-1, Ontario, Canada.	2016	Bark	4	21.16 (5.17)	*	*	*	*
PA-02	Essex-2, Ontario, Canada.	2016	Bark	3	22.13 (2.79)	*	*	*	*
PA-03	Delhi, Ontario, Canada.	2016	Bark	3	16.27 (3.61)	0.26 (0.16)	*	*	*
PA-04	Toronto, Ontario, Canada.	2016	Bark	2	18.53 (7.36)	1.10 (0.07)	*	*	*
PA-05	London, Ontario, Canada.	2016	Bark	1	22.87 (0)	0.25 (0)	*	*	*
PO-01	Experimental Farm-1, Ottawa, Canada.	2016	Bark	3	5.67 (0.35)	3.36 (0.25)	*	*	*
PO-02	Experimental Farm-2, Ottawa, Canada.	2016	Bark	3	10.81 (1.25)	2.00 (0.38)	*	*	*
PO-03	Queen Elizabeth Drive-1, Ottawa, Canada.	2016	Bark	3	9.94 (0.15)	1.36 (0.05)	*	*	*
PO-04	Queen Elizabeth Drive-2, Ottawa, Canada.	2016	Bark	3	11.41 (1.67)	0.82 (0.32)	*	*	*
PO-05	Niagara, Ontario, Canada.	2016	Bark	3	22.12 (3.69)	*	*	*	*
PO-06	London, Ontario, Canada.	2016	Bark	3	6.44 (3.68)	0.58 (0.21)	*	*	*
PO-07	Toronto-1, Ontario, Canada.	2016	Bark	3	6.67 (2.75)	1.08 (0.20)	*	*	*
PO-08	Toronto-2, Ontario, Canada.	2016	Bark	3	14.72 (1.35)	1.21 (0.13)	*	*	*
PO-09	Butlar Farms, Ontario, Canada.	2016	Bark	5	10.12 (1.31)	1.92 (0.36)	*	*	*
PO-10	Butlar Farms, Ontario, Canada.	2016	Bark	1	6.28 (0)	0.60 (0)	*	*	*
PO-11	South Windsor, Ontario, Canada.	2016	Bark	1	11.63 (0)	0.43 (0)	*	*	*
PO-12	South Windsor, Ontario Canada.	2016	Bark	1	9.91 (0)	0.74 (0)	*	*	*
PO-13	Oakland, Tennessee, U.S.	2016	Bark	3	12.85 (9.35)	0.95 (0.50)	*	*	*
PO-14	Cincinnati, Ohio, U.S.	2016	Bark	3	11.82 (6.36)	1.60 (0.13)	*	*	*
PO-15	Ocean City, Alabama, U.S.	2016	Bark	3	12.08 (3.27)	0.77 (0.18)	*	*	*
PO-16	New, Orleans, Louisiana, U.S.	2016	Bark	3	11.80 (0.21)	0.57 (0.48)	*	*	*
PO-17	Carrollton, Kentucky, U.S.	2016	Bark	3	12.42 (5.79)	0.72 (0.50)	*	*	*
PO-18	Tallahassee, Florida, U.S.	2016	Bark	1	18.34 (0)	0.68 (0)	*	*	*
PO-19	Savannah, Georgia, U.S.	2016	Bark	3	9.22 (1.04)	0.54 (0.21)	*	*	*
PO-20	North of Durham, North Carolina, U.S.	2016	Bark	1	8.02 (0)	0.42 (0)	*	*	*
PO-21	Interstate 81 Centre, Virginia U.S.	2016	Bark	1	9.53 (0)	1.20 (0)	*	*	*
PM-01	Xalapa, Mexico	2016	Bark	6	16.20 (3.22)	0.41 (0.01)	*	*	*

#: Below limit of quantification. *: Below limit of detection. SS: *Souroubea sympetala*; SG: *Souroubea Gilgii*; PA: *Plantanus x acerifolia*; PO: *Plantanus occidentalis* and PM: *Plantanus mexicana*.

However, *P. occidentalis* is the most readily available species and was the one used in the animal health product.

Finally, the analytical method was applied to determine the quantities of target metabolites in contributing *S. sympetala* herb, and *P. occidentalis* bark, used in the final tablet product intended for canine use (Table 3). *P. occidenalis* had the highest concentration of betulinic acid (over 11 mg/ g dry bark) and ursolic acid (over 1.5 mg/ g) while *S. sympetala* was a major contributor of α and β amyrin. With this blend of *S. sympetala* and *P. occidentalis* the final tablet intended for the canine market delivered 11mg of the main active principle 1 and 2.5mg of related triterpenes 2-5. As one tablet is used for a 10kg dog, it provides slightly more than the claimed 1mg/kg dose. In addition, we have completed a stability study of the tablet, and it is highly stable, for at least 1 year (data not shown). Given the results obtained in this study, this validated method is a rapid and dependable procedure to evaluate the triterpene content in different source biological materials and finished product.

Table 3: Quantification of tri-terpenes (mg/g) in raw plant material and final canine product. Mean (SEM) are presented (n=3).

Samples	1	2	3	4	5
Plant material A (*Souroubea sympetala*)	0.57 (0.05)	0.02 (0.00)	0.24 (0.01)	0.77 (0.04)	0.12 (0.00)
Plant material B (*Platanus occidentalis*)	11.73 (0.2)	0.79 (0.05)	0.04 (0.00)	0.08 (0.01)	0.00 (0.00)
Final canine product (Zentrol^{TM})	3. 22 (0.21)	0. 20 (0.01)	0.24 (0.02)	0. 12 (0.01)	0. 16 (0.00)

Experimental

Plant collection: *S. sympetala* and *S. gilgii* were collected in Costa Rica and vouchers were deposited at the herbarium Juvenal Rodriguez, Universidad Nacional (Table 2). Bark of *P. occidentalis, P. x acerifolia* and *P. mexicana* was sustainably collected in U.S, Mexico or Canada, from trunks of matures trees > 0.5 m diameter at breast height when it sheds naturally in August-September and vouchers held at the University of Ottawa herbarium.

Extraction: Extracts were prepared by Soxhlet extraction and prepared for HPLC APCI-MS as described previously [12].

HPLC-APCI-MS analyses: Solvents (LCMS grade) were from Fisher Scientific (Ottawa, ON. Canada). Authentic (>97% purity), betulinic acid, ursolic acid, lupeol, β-amyrin and α-amyrin were from Extrasynthese (Lyon, France). Targeted HPLC-MS analyses were carried out on a 3200 QTRAP (ABSciex, Concord, ON, Canada) connected to a 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, USA). Separations were performed at 1mL/min on a Kinetex C18 column, particle size 2.6-micron, 100 mm × 2.1 mm I.D. (Phenomenex, Torrance, CA, USA). Column thermostat was maintained at 55 °C during a linear gradient of 30-100% acetonitrile in water. The column was then washed with 5 column volumes with 100% acetonitrile, returned to the initial conditions in 0.1 min and re-equilibrated for 5 min before the next injection (total run time 30 min). Extracts were sonicated for 5 min and 1μL were injected in triplicate through the auto-sampler. The MS was operated in Q1MI negative ionization mode for 1 and 2 and Q1MI positive ionization mode for 3, 4 and 5 (Table 1) with a dwell time set at 100 msec. Optimal negative mode Q1MI conditions were: declustering and entrance potentials -60 V, -10 V, nebulizer current -1.0 V, source temperature 500 °C, nebulizer gases 1 and 2 set at 50 psig and 30 pisg respectively. Optimal positive mode Q1MI conditions were: declustering potential, entrance potential and nebulizer current 60 V, 10V and 3V respectively, source temperature 500 °C with ion source gases 1and 2 50 and 55 pisg respectively. Curtain gas was set at 20 L/min in both modes.

Quantification and method validation: Linear calibration curves were generated by injecting each compound using an optimized LC-MS methodology. Compounds **1**, **3-5** were quantified on the basis of area under the peak that bracketed the response obtained from the calibration range of each compound, while **2** was quantified on the basis of peak height. Precision, accuracy, and linearity thresholds were set according to ICH guidelines [14]. Recovery experiments were undertaken by spiking plant material with pure compounds at 0.1, 0.2, 0.5 and 1 mg/g of betulinic acid and 0.2, 0.4, 1 and 2 mg/g of the other metabolites were spiked; 2, 4, 10, 20 mg/g of betulinic acid were Spiked to *Plantanus* Due to the high betulinic acid concentration. Spiked and unspiked samples (in triplicate) were extracted following the procedure described above and recovery determined by regression analysis. Calibration curves were prepared at five concentration levels and R^2 values obtained for each metabolite. The limits of detection (LOD, 3:1 signal: noise) and limits of quantification (LOQ 10:1 signal: noise) were determined at 6x standard deviation of noise level. The developed and validated method was used to analyze and quantify the amount of the five selected triterpenes in plant samples as well as the Zentrol^{TM} final products.

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Conflict of interest: L Poveda, P Sanchez, M Garcia, J Baker, Z Merali, T Durst and J Arnason have direct involvement in Souroubea Botanicals Inc. which has marketed the canine product.

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