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ORIGINAL PAPER

Characterisation of steroids in wooden crates of veal calves by accelerated solvent extraction (ASE®) and ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (U-HPLC-QqQ-MS-MS)

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Abstract Illegal steroid administration to enhance growth performance in veal calves has long been, and still is, a serious issue facing regulatory agencies. Over the last years, stating undisputable markers of illegal treatment has become complex because of the endogenous origin of several anabolic steroids. Knowledge on the origin of an analyte is therefore of paramount importance. The present study shows the presence of steroid analytes in wooden crates used for housing veal calves. For this purpose, an analytical procedure using accelerated solvent extraction (ASE®), solid-phase extraction (SPE) and ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (U-HPLC-MS-MS) is developed for the characterisation of androstadienedione (ADD), boldenone (bBol), androstenedione (AED), β-testosterone (bT), α-testosterone (aT), progesterone (P) and 17α-hydroxy-progesterone (OH-P) in wood samples. In samples of wooden crates used for housing veal calves, ADD, AED, aT and P could be identified. Using the standard addition approach concentrations of these analytes were determined ranging from 20±4 ppb to 32±4 ppb for ADD, from 19±5 ppb to 44±17 ppb for AED, from 11±6 ppb to 30±2 ppb for aT and from 14±1 ppb to 42±27 ppb for P, depending on the sample type. As exposure of veal calves to steroid hormones in their housing facilities might complicate decision-making on illegal hormone administration, inequitable slaughter of animals remains possible. Therefore, complete prohibition of wooden calf accommodation should be considered.

Keywords Pressurised liquid extraction · Solid-phase extraction (SPE) · Liquid chromatography · Mass spectrometry · Steroid analytes · Wood

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Introduction

Substances exerting a hormonal action are prohibited for use in animals intended for meat production in the European Union (EU) since 1981 [1]. Currently, Council Directive 96/23/EC regulates the residue control of veterinary drugs, growth-promoting agents and specific contaminants in live animals and animal products [2].

Steroid hormones (estrogens, androgens and gestagens) may be illegally administered to meat producing animals

because of their growth-promoting characteristics [3–5]. Although the regulation on steroid abuse in livestock has long been believed to be clearly described [2], the endogenous production of specific steroid analytes by livestock under certain circumstances (stress, diet) has complicated evaluation of the results and, consequently, decision-making [6–10]. Therefore, sophisticated analytical techniques for the detection of steroid hormones and their metabolites are of main importance to decision makers. Whereas gas chromatography coupled to mass spectrometry (GC-MS^n) has long been accepted as the most powerful technique for screening and confirmatory analysis of steroids in a diversity of matrices [11–13], liquid chromatography coupled to mass spectrometry (LC-MS^n) is nowadays the most important technique of analysis [3, 5, 13–19]. As such, tedious derivatisation steps can be omitted. Moreover, the introduction of ultra-high performance liquid chromatography (U-HPLC) further allows to scale down analysis time [20].

Cattle, and in particular veal calves, have often been cited with regard to illegal administration of growth promoters such as steroid analytes [5, 13, 21]. Compared to beef, pork, lamb and poultry production and consumption, veal is a relatively minor player in the global meat industry. Nevertheless, veal is of important value to the United States and several countries of the EU (France, Italy, the Netherlands) [22–24]. To ensure a certain animal welfare, mandatory standards were enforced on the veal industry in the EU [25, 26]. As a result of these directives, a trend exists from individual housing of calves in crates towards collective housing [22, 24, 27–29]. Although materials used for the lateral partitions of the housing facilities mainly changed from wood to steel, wooden slatted floors are often maintained since they are more thermally comfortable [27, 30].

Since calves are known to spend most of their active time nibbling non-nutritive objects such as walls or bars [27, 29, 31, 32], the microbial conversion of plant-sterols or steroid precursors to steroid hormones in the wood of their housing facilities was suggested. Moreover, the policy of feeding veal calves a liquid diet has long induced nibbling on parts of their crates, whereas the availability of solid foods decreased the time spent nibbling non-nutritive objects [31]. Nowadays, as a consequence of the aforementioned directives [25, 26], the provision of a small amount of roughage in addition to a liquid diet is implied [22, 30].

Plant-sterols, structurally related to steroid hormones, are natural constituents of wood [33, 34]. A large number of studies have been devoted to their microbial conversion into steroid analytes [35–39]. In many cases, this conversion was investigated with regard to the presence of paper mill industry, processing wooden products [40, 41]. Whenever mentioned related to veal calves though, plant-sterols are suggested to be added to animal feed [5, 9, 21]. In contrast, veal calves housed in wooden crates, have always been exposed to plant-sterols. But to the best of our knowledge, no scientific literature has so far related incidences of illegal steroid administration to calves being housed in wooden crates. Nevertheless, anonymous sources reported a higher incidence of boldenone positive samples in calves housed in wooden crates versus other calves [42].

This study was conducted to investigate the presence of steroid analytes in wooden crates housing veal calves since this may be a potential source of excess steroid hormones to veal. The complexity of wood as well as the structural similarity of steroids and their low concentrations in wood, made analysis of steroids in this matrix a challenging task. Firstly, a method for fast and efficient extraction of steroid analytes from wood was developed using accelerated solvent extraction (ASE®). Secondly, fast characterisation of the steroid analytes was achieved using ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (U-HPLC-QqQ-MS-MS). Finally, these innovative techniques were applied to identify and quantify specific steroid analytes in several wood samples originating from veal calves' housing facilities.

Experimental

Chemicals and reagents

β-Testosterone (androst-4-ene-17β-ol-3-one, bT, purity ≥98%), methyltestosterone (17α-methyl-4-androstene-17β-ol-3-one, MeT, purity ≥97%), androstadienedione (androsta-1,4-diene-3,17-dione, ADD, purity ≥98%) and pregnolone (17α-hydroxy-progesterone, OH-P) were obtained from Sigma-Aldrich (St-Louis, USA). Androstenedione (androst-4-ene-3,17-dione, AED, purity ≥96%), β-boldenone (androsta-1,4-diene-17β-ol-3-one, bBol, purity ≥98%) and α-testosterone (androst-4-ene-17α-ol-3-one, aT, purity ≥98%) were purchased from Steraloids (Newport, USA). Progesterone (P) was generally gifted by the pharmacy of the Faculty of Veterinary Medicine of Ghent University. All solvents and reagents were of analytical- and HPLC-grade quality and were purchased from VWR (Merck, Darmstadt, Germany).

For each component, a primary standard stock solution was prepared in ethanol at a concentration of 200 $ng\,\mu L^{-1}$. Working standard solutions were prepared by appropriate dilution of these stock solutions in ethanol. All standard solutions were stored at 4°C following the quality assurance instructions of Belac accreditation (EN17025).

Sample preparation

As tabulated in Table 1, a selection was made of wood species of different origin. In all the selected wood species, a clear distinction between sapwood and heartwood was shown. Of all wood species only the heartwood was used to prepare the samples for this study. Prior to preparing small wood shavings, the massive batons of wood were planed. As such, only fresh heartwood was taken for this experiment, excluding influences of dust particles or chemical components due to oxidative ageing of the wood surface.

In addition to these samples, wood originating from calves' crates, identified as *Lophira alata*, was examined (Samples A, C and D). One of these samples originated from a Dutch veal farm, the other from a Belgian farm. Contamination of the planks with hair and faeces was considered as a separate sample, referred to as the top-wood sample (Sample B). The same analytical procedure was applied for analysis of this matrix as the one developed for analysis of wood samples.

A plane (C2500/3L DX T.400, Sicar) was used to obtain small wood shavings from the prepared batons. Rotation speed was set at 5,200 tours per minute (tpm) with a feeding speed of 10 m/min. The wood dust collector of the plane was diverted to capture the shavings in a separate dust bag. Between the sampling of the selected wood species, the entire instrument, including the dust suction collector, was cleaned carefully by high pressured air.

Extraction and clean-up

Pressurised liquid extractions were performed on a Dionex ASE® 350 Accelerated Extractor with Solvent Controller (Dionex Corp., Sunnyvale, CA, USA). One gramme of ground wood was dispersed in one gramme of diatomaceous earth (DE, ASE® Prep Diatomaceous Earth, Dionex Corp.). A cellulose filtre (27 mm, Dionex Corp.) was placed on the bottom of a 22-mL stainless steel extraction cell. Each cell was filled with 5 g of aluminum oxide 90 aktiv neutral (Dionex Corp.) before addition of the sample mixture. Extraction was carried out at 100 °C using acetone/methanol (2/1) as extraction solvent. After filling of the cell with solvent, a pressurised static extraction phase lasting 15 min was performed, followed by a flow of fresh solvent. The extract was evaporated under nitrogen at 60±2 °C prior to solid-phase extraction (SPE). Based on the method described by Impens et al. [11], a combination of silica (Si) cartridges (Isolute® 500 mg 10 mL SPE columns, Biotage AB, Uppsala, Sweden) and aminopropyl-functionalised silica (NH_2) cartridges (Isolute® 100 mg 1 mL SPE colums, Biotage AB) was used for optimal SPE procedure. All analytes were eluted using chloroform/acetone (4/1). Prior to U-HPLC-MS analysis, elutes were evaporated once again under nitrogen at 60±2 °C to be subsequently reconstituted in methanol diluted with aqueous formic acid and centrifuged at 9,000 tpm for 10 min at 4 °C.

U-HPLC-MS analysis

The U-HPLC apparatus comprised of an Accela™ High Speed LC and an Accela™ autosampler (Thermo Electron Corporation, San Jose, USA). To achieve chromatographic separation, several columns were tested, a Hypersil Gold™ column (1.9 µm, 50×2.1 mm, Thermo Electron), a Nucleodur C18 Isis (1.8 µm, 50×2.1 mm, Macherey-Nagel, Düren, Germany), a Nucleodur C18 Gravity (1.8 µm, 50×2.1 mm, Macherey-Nagel), a Nucleodur Sphinx RP (1.8 µm, 50×2.1 mm, Macherey-Nagel) and an Acquity HSS T3 (1.8 µm, 50×2.1 mm, Waters, Milford, USA). An Acquity UPLC™ in-line filtre (2.1 mm, 0.2 µm, Waters) was used to improve analytical column lifetime. The mobile phase constituted of 0.1% formic acid in methanol and 0.1% aqueous formic acid. Optimised separation of all analytes was obtained on a Nucleodur Sphinx RP (1.8 µm, 50×2.1 mm, Macherey-Nagel) using a linear gradient starting with a mixture of 35% aqueous formic acid and 65% formic acid in methanol. The methanol percentage increased from 65% to 95% in 2 min. Between samples, the column was allowed to equilibrate at initial conditions for 1 min. All analytes could be separated in a total runtime of only 6 min.

Table 1 Overview of the trivial names, respective botanical names, families and natural growth area of selected wood species

Wood name	Botanical name	Family	Natural growth area
Angelim vermelho	*Dinizia excelsa*	*Mimosaceae*	South America
Azobe	*Lophira alata*	*Ochnaceae*	Western and Central Africa
Douglas	*Pseudotsuga menziesii*	*Pinaceae*	Europe
Massaranduba	*Manilkara bidentata*	*Sapotaceae*	South America
Oaken	*Quercus sp.*	*Fagaceae*	Europe
Oregon	*Pseudotsuga menziesii*	*Pinaceae*	West of North America
Pine	*Pinus* sp.	*Pinaceae*	Europe
Western Red Cedar	*Thuya plicata*	*Cupressaceae*	West of North America

Table 2 Default and optimised HESI-II working parameters for ionisation of specific steroid analytes

	Default	Optimised
Spray voltage (V)	3,000	3,000
Capillary temperature (°C)	250	310
Sheath gas pressure (psi)	30	45
Auxiliary gas pressure (arbitrary units, au)	30	15
Heater temperature (°C)	325	370

Detection was carried out using a TSQ Vantage Triple Stage Quadrupole Mass Spectrometer (Thermo Electron) equipped with a heated electrospray ionisation probe (HESI-II). The parameters as presented in Table 2 were found to be the optimal ionisation source working parameters for the respective analytes.

All analytes were detected in positive ion mode selected reaction monitoring (SRM). An overview of the transition specifics for all analytes is provided in Table 3. The mass resolution at the first (Q1) and third (Q3) quadrupole was set to 0.7 Da at full width at half maximum (FWHM). The cycle time was adjusted to 1.7 s, the collision gas pressure was set at 1.5 mTorr and the chrome filtre peak width at 5 s.

Quality assurance

Prior to sample analysis, standard mixtures of the targeted analytes were injected in order to check the operation conditions of the chromatographic devices. To every sample, a procedure internal standard (IS), MeT was added prior to extraction. Different analytes were identified by comparison of their retention time relative to the IS, with that of standards. In addition, the ion ratios of the product ions were compared with those of standards. All investigated analytes were characterised by a unique relative retention time and specific ion ratio of the product ions, showing good selectivity of this analytical method. The specificity of the method was demonstrated by analysis of non-fortified *Quercus* sp. samples and samples fortified with the analytes of interest at a concentration of 100 ppb. For each analyte spiked, the chromatogram showed a significant increase in peak area and intensity at its specific retention time compared to blanks, taking a signal-to-noise ratio of at least three into account. Repetitive injection of a mixture of all analytes in standard solution (n=10), at a concentration of 0.1 ng on column, showed good repeatability of the method with a standard deviation in the range of 0.2–1.0% and 2.5–7.0%, respectively, on the relative retention time and the area ratio. After identification, the analyte's concentration was estimated by fitting its area ratio in an eight-point calibration curve established in standard solution. Area ratios were determined by integration of the area of an analyte under the specific SRM chromatograms in reference to the integrated area of the internal standard. The instrument's limit of detection, determined by standard injection with a signal-to-noise ratio of at least 3 was 5.0 pg on column for all analytes of interest. For every single analyte, correlation coefficients obtained for the calibration curves were higher than 0.97, showing good linearity in the range of 0.1–10.0 ng on column, and the IS at 5.0 ng on column. To quantify analytes, the standard addition approach was the method of preference.

Standard addition approach

The standard addition approach used in this study was based on the approach mentioned in Commission Decision 2002/657/EC [43]. After extraction and clean-up, dried

Table 3 Selected reaction monitoring specifics for all analytes of interest: protonated molecules at appropriate S-Lens RF-amplitude, and the corresponding collision energy (CE) necessary for fragmentation into specific fragments

Analyte	Molecular molecule (m/z)	Product ion (m/z)	CE (V)	S-Lens (V)
ADD	285.15	77.16	51	54
		91.15	39	
		121.16	22	
		147.19	15	
bBol	287.17	77.16	51	56
		121.17	26	
		135.24	17	
AED	287.19	79.17	36	70
		81.18	37	
		97.18	21	
		109.17	25	
bT	289.20	79.15	39	70
		97.16	22	
		109.15	27	
		253.32	15	
P	315.21	79.17	38	75
		97.15	23	
		109.15	28	
		297.33	13	
aT	289.20	79.17	40	70
		97.16	23	
		109.16	27	
		253.30	16	
MeT	303.22	97.17	30	73
		109.16	28	
		285.35	15	
OH-P	331.15	79.15	45	77
		97.16	28	
		109.14	30	
		295.32	17	

residues were divided into two aliquots of analogous mass (m) and volume (V). One aliquot, the unknown, was reconstituted in methanol/aqueous formic acid (35/65) for injection on the U-HPLC-MS-MS system. After analysis of the unknown, the concentration of each identified analyte was estimated by fitting its area ratio, x_{unknown}, in a calibration curve set-up in standard solution. Based on this estimation, the other aliquot was spiked with a similar known concentration of the identified analyte (A). Final reconstitution of this aliquot was also performed in methanol/aqueous formic acid (35/65). U-HPLC-MS-MS analysis of this aliquot resulted in an area ratio of x_{known}. Using the following formula, the unknown concentration (C_{unknown}) was calculated:

$$C_{\text{unknown}} = x_{\text{unknown}}\, V_{\text{unknown}}\rho_A V_A / (x_{\text{known}} V_{\text{known}} m_{\text{unknown}} - x_{\text{unknown}} V_{\text{unknown}} m_{\text{known}})$$

with $V_{\text{unknown}} = V_{\text{known}}$ and $m_{\text{unknown}} = m_{\text{known}}$

$$C_{\text{unknown}} = x_{\text{unknown}}\rho_A V_A / (x_{\text{known}} - -x_{\text{unknown}})$$

C concentration
x area ratio
V volume
ρ concentration
m mass
A identified analyte

Data analysis

Data were interpreted using Xcalibur 2.07 software (Thermo Electron, San José, USA). Statistical analyses were performed with the software package Statistica™ (Statsoft, Tulsa, USA). Data were checked for normality and homogeneity of variances using Kolmogorov–Smirnov and Levene's test, respectively, both with an α-error of 0.05. The effect of a parameter was tested for significance using a one-way analysis of variance (ANOVA, Dunnett's test).

Results

ASE optimisation

Several parameters of the ASE procedure were optimised by analysis of *Quercus* sp. samples spiked at 200 ppb of ADD, AED, bBol, bT, P, aT, and OH-P while the internal standard, MeT, was spiked at 500 ppb. Spiking was done on the cellulose filtre, resulting in higher recoveries with better reproducibility than spiking on top of the sample (Fig. 1). Apparently, the absorbance characteristic of diatomaceous earth has a potential negative effect on spiking.

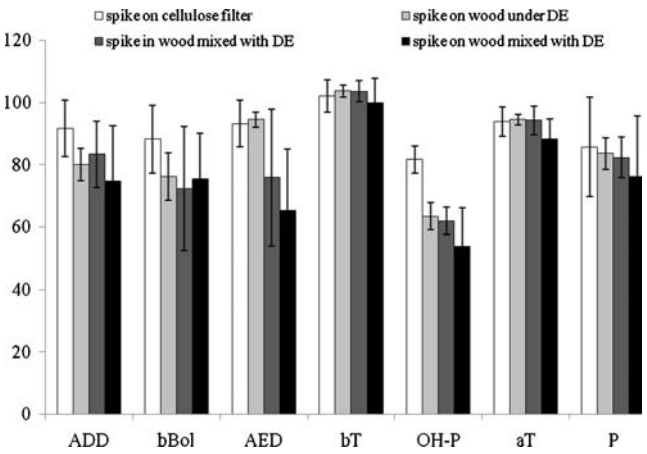


Fig. 1 Mean recoveries (%) of steroid analytes (ADD, bBol, AED, bT, OH-P, aT, P) spiked at 200 ppb in *Quercus* sp. *Error bars* correspond to standard deviations on the mean, with n=5. Different *coloured bars* represent different ways of spiking

First, extraction solvents with different polarity characteristics were tested (*n*-hexane, diethyl ether, ethyl acetate, methanol, acetone/methanol (2/1)). Second, temperature (60–100–140–180 °C), static time (5–10–15–20 min), flush volume (20–40–60–80%) and the number of extraction cycles (three times 5 min–two times 10 min–one time 15 min) were evaluated. The average area of all targeted analytes was used as a marker to evaluate the extraction efficiency. This way, the default ASE extraction procedure as provided by the manufacturer was optimised for the extraction of steroid analytes from wood by evaluating one individual parameter at a time (Table 4).

The most optimal extraction solvent was selected based on analytical characteristics such as peak area and signal-to-noise ratio, but also on visual characteristics such as lightness and contamination of the obtained extract. According to these criteria, extraction with methanol or acetone/methanol (2/1) led to the best results, giving significantly higher peak areas than with *n*-hexane ($p<0.01$), diethyl ether ($p<0.01$) or ethyl acetate ($p<0.05$). Since peak areas were slightly higher and samples clearly less contaminated using acetone/methanol (2/1), this solvent was selected for further experiments.

Extraction efficiency at a temperature of 180 °C was significantly lower compared to extraction at a temperature of 140 °C ($p<0.01$), 100 °C ($p<0.05$) or 60 °C ($p<0.01$). No significant differences could be observed between 140 °C, 100 °C or 60 °C. A static time of 5 min showed significantly lower extraction efficiency than a 20 min static time ($p<0.05$). Average peak areas of all analytes extracted using static times of 10 or 15 min were higher than in the case of a 5-min static time and lower than in the case of a 20-min

static time, although not significantly. Whether this static time of 15 min was executed using one cycle of 15 min, two cycles of 10 min or three cycles of 5 min did not result in significant differences. An increase of the flush volume on the other hand from 40% to 60% significantly increased peak areas. No significant differences were shown between a flush volume percentage of 20%, 60% or 80%, but peak areas were slightly higher at 60%.

As a result of these experiments, optimised working conditions for extraction were one extraction cycle of 15 min at 100 °C with a flush volume of 60% (Table 4).

Clean-up optimisation

As both reversed-phase (RP) SPE and normal phase (NP) SPE have been described in literature for the clean-up of steroids extracted from solid matrices [5, 11, 44], the optimal SPE retention mechanism (RP or NP) for this particular application was identified by evaluating both mechanisms. For this purpose, RP extraction using octadecyl end-capped functionalised silica (C18) cartridges was compared to NP extraction using silica (Si) cartridges (Table 5). For all analytes, peak areas were higher when applying the NP mechanism, using Si as specific sorbent, than when performing RP extraction. To provide an additional clean-up, these cartridges were combined with aminopropyl-functionalised silica (NH_2) cartridges as described by Impens et al. [11].

Chromatography

Chromatography of all steroid analytes was optimised comparing different columns, column temperatures, flow rates and injection volumes. For this purpose, a mixture of all analytes dissolved in the initial gradient solution was injected so that a final amount of 100 pg was brought onto the column. The choice of the column was mainly determined by the peak efficiency, measured as peak width at the baseline, and the separation efficiency for structurally related steroid analytes.

Peaks were clearly smaller using a Nucleodur Sphinx RP column under the same operating conditions. Mean peak widths for ADD using a Nucleodur Sphinx RP column were 0.43±0.02 min, while widths ranged from 0.47±0.07 min to 0.59±0.05 min using other columns. Base peak separation of bBol and AED, and aT and bT within a runtime of 6 min could also best be achieved using the Nucleodur Sphinx RP column. For these reasons, this column was selected for further optimisation.

The influence of column temperature (30–60–90 °C), flow rate (200–300–400–500–600 µL min^{-1}) and injection volume (2–5–10 µl) on chromatography was further evaluated.

Based on the peak width, peak height and signal-to-noise, best peak performance was achieved at a column temperature of 30 °C, a flow rate of 200 µL min^{-1} and an injection volume of 5 µL. In Fig. 2, chromatograms are displayed of all analytes analysed under these conditions.

Mass spectrometry

Starting from the default parameters for the HESI-II probe as provided by the manufacturer, capillary temperature, sheath gas pressure, auxiliary gas pressure and heater temperature were further optimised by infusion of the analytes' mixture. An overview of the optimised parameters for the HESI-II probe is provided in Table 2.

In order to establish appropriate selected reaction monitoring for the individual steroid analytes, standards diluted in the mobile phase were infused into the mass spectrometer. Collision energy (CE) of each protonated molecule was adjusted for the most abundant product ions. For each analyte, at least three transitions were retained for identification. S-lens RF-amplitude voltage was evaluated for each specific parent ion and collision energy was evaluated for each transition independently. Results are summarised in Table 3.

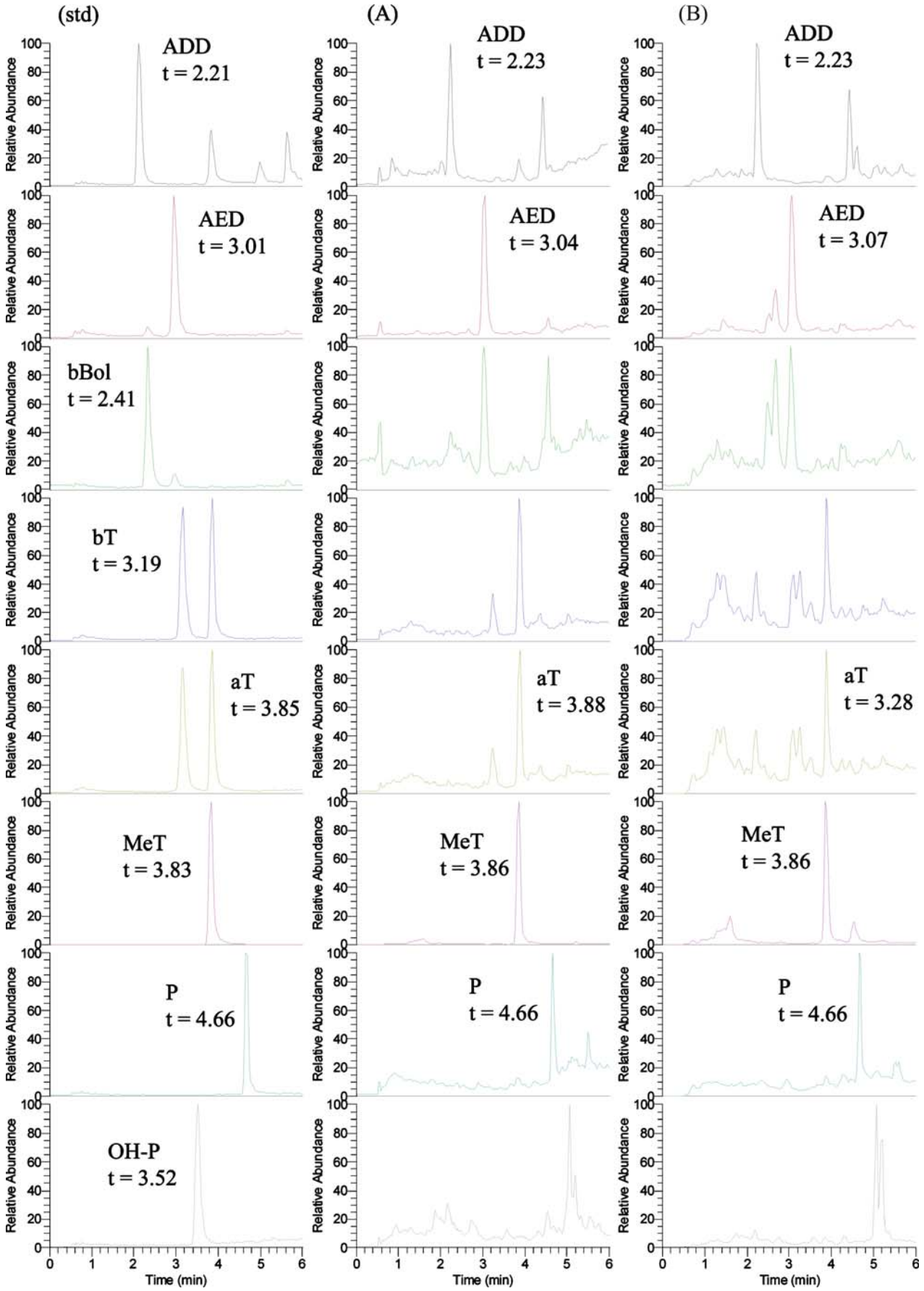
Table 4 Default and optimised working parameters for ASE extraction of steroid analytes from wood

	Default procedure	Optimised procedure
Temperature (°C)	120	100
Static time (minutes)	15	15
Flush volume (%)	60	60
Number of cycles	1	1

Table 5 Different steps of SPE procedure following reversed-phase (RP) retention or normal phase (NP) retention

	RP (C18)	NP (Si)
Sample pre-treatment	Water	Optional
Sorbent solvation	Methanol	*n*-hexane
Sorbent equilibration	Water	*n*-hexane
Sorbent wash	Water	*n*-hexane
Elution	Methanol	Chloroform/acetone (4/1)

Fig. 2 Chromatograms of ADD, AED, bBol, bT, aT, MeT, P and OH-P for a standard mixture (*std*, 100 pg on column), a *L. alata* sample from a calves' crate (*A*) and the respective top-wood sample (urine, faeces, etc.) of sample A (*B*) ▶


(std)
(A)
(B)
ADD
t = 2.21
ADD
t = 2.23
ADD
t = 2.23
AED
t = 3.01
AED
t = 3.04
AED
t = 3.07
bBol
t = 2.41
bT
t = 3.19
aT
t = 3.85
aT
t = 3.88
aT
t = 3.28
MeT
t = 3.83
MeT
t = 3.86
MeT
t = 3.86
P
t = 4.66
P
t = 4.66
P
t = 4.66
OH-P
t = 3.52
Relative Abundance
Time (min)

Characterisation of steroids in wood samples

This analytical method was originally developed to identify steroids in wood samples. The limit of detection (LOD) was determined in *Quercus* sp. by spiking the analytes of interest. Signal-to-noise ratio resulted to be at least 3 for ADD, AED, bBol, aT and P at a concentration of 5 ppb. For bT and OH-P the LOD was 10 ppb. Nevertheless, attempts were made to quantify the identified steroids as well. Recoveries for steroid extraction were determined in a single wood species *Quercus* sp. by comparing the measured area ratio of a known concentration of an extracted analyte with the area ratio of a standard solution corresponding to that concentration (200 ppb). As presented in Fig. 1, recoveries using the optimised analytical procedure were 92±9% for ADD, 88±11% for bBol, 93±7% for AED, 102±5% for bT, 86±16% for P, 94±5% for aT and 82±4% for OH-P. For all analytes, good recoveries were achieved when extracting *Quercus* sp. samples. However, analysis of different wood species revealed a non-negligible influence of the matrix on the recoveries. For this reason, the standard addition approach, compensating for differences in the wood matrix, was chosen for quantification of analytes identified in wood samples.

From the different species of wood investigated in this study (*Dinizia excelsa*, *L. alata*, *Pseudotsuga menziesii* (Europe), *Manilkara bidentata*, *Quercus* sp., *P. menziesii* (West of North America), *Pinus* sp. and *Thuya plicata*), only *Quercus* sp. resulted to contain a naturally occurring steroid, progesterone (Fig. 3). The concentration was estimated to be 14±1 ppb (n=6). According to the standard addition approach this concentration was calculated to be 19±6 ppb (n=6).

Whereas none of the analytes of interest could be detected in *L. alata* heartwood, analysis of *L. alata* samples originating from wooden crates in which veal calves had been housed, showed the presence of ADD, AED, aT and P independent on the farm at which they were collected (Table 6). In Fig. 2, identification of these analytes in a wood sample (A) and a top-wood sample (B) is shown in comparison with a standard solution.

Wood analysis resulted in similar concentrations for all analytes for the Dutch and the Belgian wood samples (A). In the case of the top-wood samples (B), concentrations of

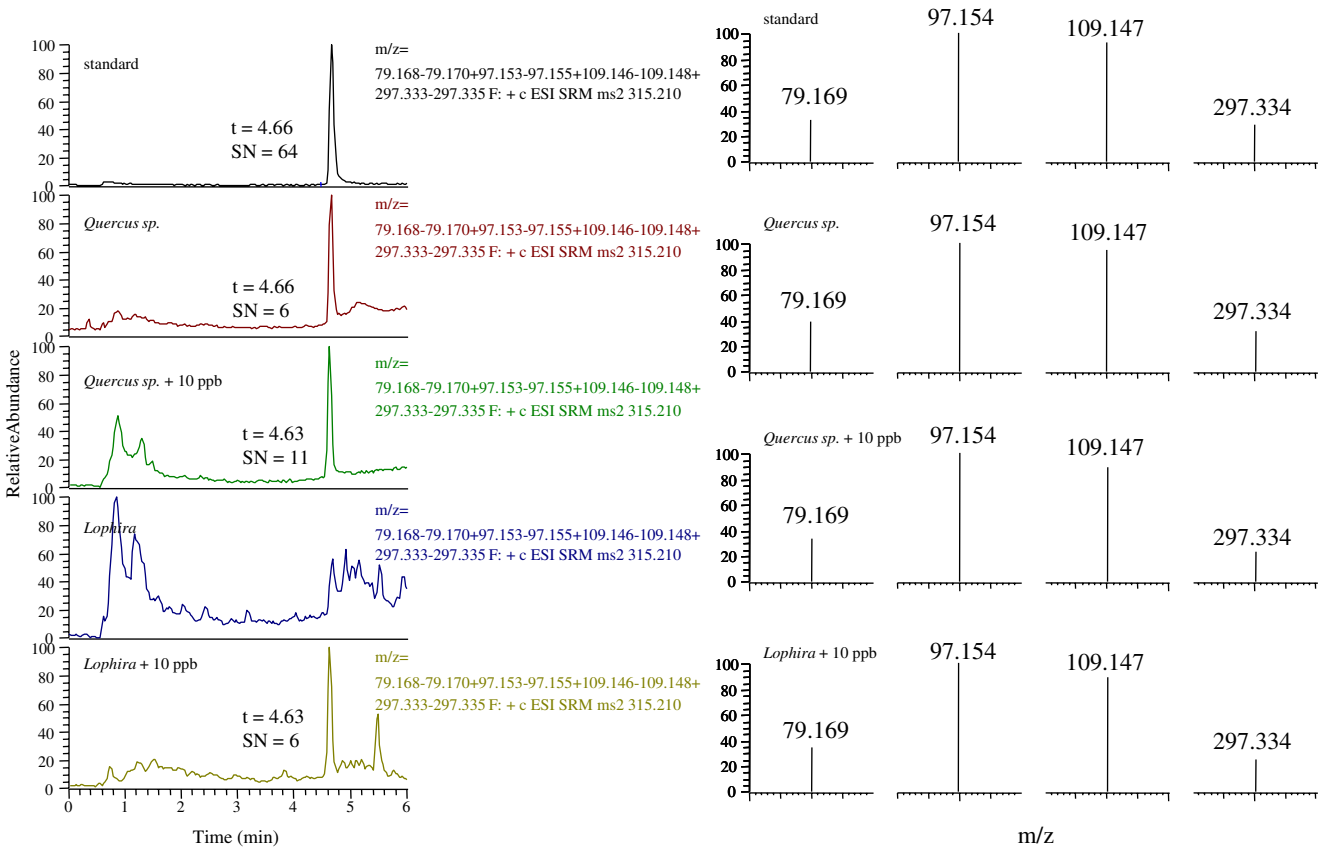


Fig. 3 Chromatograms and spectra of progesterone in a 100 pg standard solution, a non-fortified *Quercus* sp. sample, a 10-ppb-fortified *Quercus* sp. sample, a non-fortified *L. alata* sample and a *L. alata* sample from a wooden crate

Table 6 Concentrations of steroids (mean±standard deviation in parts per billion) in *Lophira alata* originating from wooden crates of a Dutch (d) and Belgian (b) farm, quantified using the standard addition approach, and estimated using standard calibration curves

	ADD	AED	aT	P
Sample A[a]-d (n^n=4)	24±4	33±8	11±6	14±1
	41±12	*54±16*	*11±5*	*13±2*
Sample B[b]-d (n^n=1)	60	68	35	–
	127	*104*	*28*	*68*
Sample A[a]-b (n^n=4)	20±4	19±5	16±6	14±3
	35±4	*33±7*	*14±0*	*10±2*
Sample B[b]-b (n^n=2)	26±3	35±9	30±2	42±27
	61±2	*45±2*	*20±2*	*18±1*
Sample C[c]-b (n^n=3)	22±2	44±17	13±1	35±19
	38±1	*78±42*	*15±3*	*18±5*
Sample D[d]-b (n^n=3)	32±4	<10	–	<10
	50^s	*<10*^{s}	–	*<10*^{s}

Values in roman are concentrations quantified using the standard addition approach. Values in italics are concentrations estimated using the standard calibration curves

n number of replicates, *s* only one replicate

[a] First plus second millimetre top layer of wooden plank

[b] Contamination (hair, faeces, etc.) eliminated from plank

[c] First millimetre top layer of wooden plank

[d] Third plus fourth millimetre top layer of wooden plank

ADD, AED and aT were about twice the concentrations detected in wood samples (A). Nevertheless, progesterone could not be detected in the top-wood sample of the crate originating from the Dutch farm, while its concentration was variable in the analogous sample derived from the Belgian farm. It could be expected that the analyte concentrations decreased from the outer to the inner layer of the wooden planks. For AED and P the outer concentrations (C) were higher than the inner concentrations (D). In contrast, this trend could not be observed for ADD and aT.

A comparison was made between the concentrations obtained using the standard addition approach and those estimated using appropriate calibration curves in standard solution (Table 6). For ADD and AED, an overestimation could be observed for the concentrations estimated using calibration curves. This lies within the expectations since matrix effects are not considered using the latter approach. The difference in quantification approach was less prominent for the concentrations of aT and P. Except that, based on the calibration curves, a P-concentration of 68 ppb was established in the top-wood Dutch sample (B) while this concentration could not be quantified using the standard addition approach.

Discussion

The results obtained in this study indicate the presence of steroids in wooden crates, which may lead to an excess steroid exposure to veal calves. Hence, this study is of important value to the discussion on the endogenous production of steroid analytes by veal. These results could only be achieved however, based on an analytical method allowing fast and efficient analysis of steroids in wood samples.

Accelerated solvent extraction (ASE®) has already been described by several authors as a means to extract a diversity of analytes from wood samples [45–48]. However, in unravelling the composition of wood, these studies consider analytes of interest other than steroids.

Steroids cover, dependent on the functional groups on their steroid nucleus, a rather wide range of polarity. For this reason, selection of an appropriate extraction solvent is of crucial importance. ASE® allows conventional extraction solvents to be used. In accordance with Impens et al. [11], extraction efficiency of steroids was significantly better using methanol or a mixture of methanol and acetone compared to less polar solvents. Whereas a large amount of organic solvent is required when using traditional methods to extract steroids from wood, elevated solvent temperatures used during ASE result in greater sample penetration capabilities and better extraction efficiencies and consequently lower solvent usage. Moreover, the combination of elevated extraction temperatures and pressures increases the kinetics of the extraction process, as such decreasing the extraction time [46, 49]. For this application, extraction times above 15 min did not significantly alter the extraction efficiency.

To avoid interferences with low steroid contents detected in complex wood samples, a clean-up procedure was required. The most favourable results were obtained using Si cartridges in combination with NH_2 cartridges. Retention of an analyte under normal phase conditions is primarily due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface. As such, the unbounded silica surface of the Si cartridge allows for hydrogen bonding and π–π interactions among others, which gives strong retention for relatively polar compounds similar in structure such as steroids [50]. Final detection of steroids was performed using a fast and effective U-HPLC-QqQ-MS-MS method.

Over the last decades, the analysis of steroids underwent an evolution from laborious GC analysis towards fast LC analysis [3, 5, 11–19]. Recently, the introduction of U-HPLC and UPLC even further decreased the analysis time required for steroid determination [51–53]. The latter techniques allow optimised performance and improved chromatography provided by the sub 2-μm particle columns, allowing increased separation efficiency, high sensitivity and decreased analysis time [53].

With regard to retention and selectivity, the choice of an appropriate column for steroid analysis is besides steroids' polarity also dependent on the molecular shape. Steroids are structurally very similar so that enhanced shape selectivity may be an important consideration when choosing a suitable system for their separation. Although Hypersil Gold™ columns allowed acceptable separation efficiency, columns with different reversed-phase packings were tested in order to improve the separation of bBol and AED, and bT and aT in particular. The unique concept of the Nucleodur Sphinx RP incorporates steric selectivity, in other words the ability to separate analytes in relation to their molecular structure and geometry. The unique selectivity surface existing of a bi-functional phase of octadecyl (C18)- and phenylpropylsilanes, combines both hydrophic and π–π interactions retaining the aromatic moieties of steroid molecules.

A methanol gradient was applied to cover the range of required solvent strength necessary to elute all steroid analytes of interest. The elution of steroids is dependent on the number and the nature of their polar functional groups and non-polar residues. Because of the absence of polar hydroxyl (OH)-groups in ADD- and AED-molecules for instance, minor retention was shown and low amounts of methanol in the mobile phase were sufficient for elution of these analytes. The incorporation of U-HPLC enabled the elution of eight structurally closely related steroids in a runtime of only 6 min.

The described analytical method allowed the identification of ADD, AED, aT and P in wood samples. Interestingly, the analysis of wood samples of trees of diverse origin only showed the presence of P, and only in one species of wood *Quercus* sp. (Fig. 3). Nevertheless, naturally occurring progesterone has been previously reported in wood [38, 54]. Although Carson et al. [40] mentioned *Pinus taeda* to be a rich source of naturally occurring progesterone, concentrations in the leaves of *P. taeda* were about 1,000 times higher than the concentrations obtained in our study for *Quercus* sp. This might be explained however by the change in steroid content during plant development and its dependence on the species and cultivar [55]. In addition, these authors reported that, besides P, also testosterone and AED have been detected in many plants and frequently their involvement in the physiology of plants has been evidenced. For three plant species (*Digitalis purpurea*, *Nicotiana tabacum*, *Inula helenium*) Simersky et al. [56] also reported the presence of P and AED, as well as OH-P and 16-dehydroprogesterone.

In accordance with previous results, we also demonstrated the presence of P, AED and aT in wood samples originating from veal calves' crates. To the best of our knowledge, this study is the first to report the characterisation of ADD in wood samples. The microbial conversion of progesterone to ADD and AED however, has been previously described [37, 40], indicating that progesterone may function as immediate precursor for androgen biosynthesis. This occurrence of P has so far been attributed to microbial conversion of plant-sterols, natural constituents of wood [41, 57]. Nevertheless, up until now, the conversion of P into ADD had not been established in wood. Indeed, it is a striking finding that only in wood samples from veal calves' housing facilities, ADD could be characterised. Concentrations of ADD in these wood samples were in the low-ppb range (<100 ppb).

Explanations on the conversion of P or other steroid precursors to ADD in wood can possibly be found in the crucial role specific enzymes play. As veal spread urine and faeces in their crates, the necessary microorganisms for that conversion might be available. Another source of metabolic activity might be provided by the veal calves by nibbling parts of their housing. About 22% of the daytime of veal calves is spent on nibbling [40]. Furthermore, this oral activity is also a potential route of exposure of veal calves to an excess of steroid analytes, serving as precursors or intermediary products for bioconversion to active androgens. As a result, endogenous androgen production by veal calves is likely to occur. As this might have implications for regulatory affairs on illegal administration of steroids as growth promoters, the prohibition of wooden crates as housing facilities for calves should be recommended.

Conclusion

In this study, an analytical procedure was developed for the characterisation of selected steroids in a wood matrix. Extraction of steroids from a complex matrix such as wood in the low-ppb range was achieved by a combination of accelerated solvent extraction and appropriate solid-phase extraction. Sensitive detection in a total runtime of only 6 min was obtained by U-HPLC-QqQ-MS-MS analysis.

Using this procedure, the characterisation of ADD in *L. alata* wood, originating from wooden crates used for housing veal calves, is reported. Moreover, several other steroids, in particular AED, aT and P, could be characterised in these samples.

Evidence on the presence of steroids in wooden crates might be of important value to decision makers, since exposure of veal calves to these analytes might induce endogenous production of active androgens. To prevent animals from inequitably being slaughtered because of the suspicion of illegal hormone administration, wooden calf accomodation should be avoided.

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