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Impact of extraction solvents on steroid contents determined in beef

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Abstract

Steroid hormone contents in polar and non-polar extracts from beef determined by GC–MS were compared. Polar extraction of beef was carried out with a mixture of methanol–water as usual in steroid analysis. The residue of polar extraction was extracted sequentially with *n*-hexane and ethyl acetate and were combined to form the non-polar extract. The polar extract as well as the non-polar extract were purified separately by liquid–liquid extraction followed by solid phase extraction. Eight beef samples were analysed for their steroid hormone contents after polar and non-polar extraction. Furthermore, their fat content was determined. Steroid hormone contents in the non-polar extract could not be neglected. Consequently, the usual polar extraction methods for the determination of anabolic steroids are insufficient. Steroid hormone patterns of polar extracts and of non-polar extracts are comparable. A comparison between steroid contents determined by different extraction methods is not possible.

1. Introduction

The determination of steroid hormone contents and patterns of beef with GC–MS is carried out after an extraction with a mixture of methanol–water by several authors and is also used for the German reference method (Bergner-Lang & Kächele, 1981; Bundesgesundheitsamt—Arbeitsgruppe "Anabolica" nach § 35 LMBG, 1989; Fritsche, Schmidt, & Steinhart, 1999; Verbeke, 1979). For determination by radioimmunoassay, meat samples are homogenised with a buffer and extracted from the buffer with non-water soluble solvents (Henricks & Torrence, 1983; Hoffmann, 1983; Meyer, 1990; Sjövall & Axelson, 1982). Polar solvents as methanol–water or buffers are preferred for steroid extraction because extraction with non-polar solvents leads to fatty extracts, and consequently, to problematic clean up procedures.

However, steroid hormones are soluble in polar and non-polar solvents. They accumulate in adipose tissue, which acts as a steroid reservoir (CEC, 1989; Fehér & Bodrogi, 1982; Gaiani & Chiesa, 1986; Wähner, Schnurrbusch, Engelhardt, Gottschalk, Scharfe, & Pfeiffer, 1993). Storage of steroids in fat cells of muscle tissue seems to be also possible. An increased level of steroid hormones could be found in beef samples with a high level of intramuscular fat (Fritsche, Schwarz, Kirchgeβner, Augustini, & Steinhart, 1998). Steroids embedded in muscle cells with high fat content are not considered by the usual polar extraction methods because polar solvents are not able to penetrate hydrophobic structures in tissues. Storage forms of steroids as fatty acid esters (Hochberg, Pahuja, Larner, & Zielinski, 1990; Mellon-Nussbaum, Ponticorvo, Schatz, & Hochberg, 1982) are also not extracted because of their non-polarity. Steroid fatty acid esters are lipoidal derivatives of steroids which are esterified often at C 17 with fatty acids (Ardévol et al., 1997; Larner, MacLusky, & Hochberg, 1985; Pahuja, Zielinski, Giordano, McMurray, & Hochberg, 1991). Their occurrence in muscle tissue is not examined.

Therefore, it is questionable if the polar extraction method generally used includes all steroids. Due to reasons mentioned above, polar extraction followed by a non-polar extraction step should be superior to the polar extraction method. It seems to be possible that extraction with methanol–water considers more polar steroids than less polar steroids.

In the present study, the content and the pattern of steroid hormones in beef determined after a polar and a

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non-polar extraction were examined and compared. The intramuscular fat content was examined to assess its dependence on steroid contents.

2. Materials

2.1. Steroids

Androstenedione (4-androstene-3,17-dione), androsterone (5α-androstane-3α-ol-17-one), testosterone (4-androstene-17β-ol-3-one), and progesterone (4-pregnene-3,20-dione), were purchased from Serva (Heidelberg, Germany). Dehydroepiandrosterone (DHEA, 5-α-androstene-3β-ol-17-one), dihydrotestosterone (5α-androstane-17β-ol-3-one), epitestosterone (4-androstene-17α-ol-3-one), hydroxyprogesterone (4-pregnene-17α-ol-3-one), pregnenolone (5-pregnene-3β-ol-20-one), and methyltestosterone (17α-methyl-4-androstene-17β-ol-3-one were obtained from Sigma (Deisenhofen, Germany).

2.2. Reagents

Methanol, ethanol, *n*-hexane, ethyl acetate, diethyl ether, chloroform, methyl-tert-butyl ether (MTBE), potassium hydroxide, sodium methylate, 1,4-dithioerytritol, methylene chloride and N-methyl-N-trimethylsilyltrifluoroacetamide were from Merck (Darmstadt, Germany) in analytical grade. Trimethyliodosilane (TMIS) was from Fluka (Neu-Ulm, Germany). Solid-phase extraction (SPE) cartridges (Bond Elut C_8, Bond Elut Si and Bond Elut NH_2, 500 mg, 3 ml each), 20 ml polypropylene reservoirs and a vacuum manifold were kindly donated by Varian (Darmstadt, Germany).

2.3. Samples

The meat samples were taken from the loin (*longissimus dorsi* muscle) from Charolais steers of the same age and average slaughter weight of about 200 kg. They were stored at −20 °C until analysis and were homogenised with a household mixer immediately before analysis.

3. Methods

3.1. Extraction of free steroids with polar solvents

Free steroids were extracted from 20 g beef with a mixture of 70 ml methanol and 20 ml water as described previously (Fritsche et al., 1999; Hartmann & Steinhart, 1997). The extract was defatted twice with 20 ml *n*-hexane and cleaned up by solid phase extraction using a C_8-cartridge. The elute was extracted with diethyl ether to remove the estrogens and the residue was filtered through a silicagel-cartridge. The elute was dried, silylated to trimethylsilyl ethers and analysed by GC–MS in the selected ion monitoring mode (Fritsche et al., 1999; Hartmann & Steinhart, 1997).

3.2. Extraction with non-polar solvents

Ethyl acetate (50 ml) was added to the residue of the methanol–water extraction. The mixture was homogenised with a rod homogeniser (e.g. Ultra-Turrax, Jahnke und Kunkel, Staufen, Germany) and centrifuged at 2000 *g* for 10 min. The supernatant was collected in a round-bottomed flask. *n*-Hexane (50 ml) was added to the residue. The mixture was homogenised as the ethyl acetate extract. The supernatant was combined with the ethyl acetate extract and with the *n*-hexane resulting from the defattening step of the methanol–water extraction. Solvents were evaporated by a vacuum evaporator (40 °C). The sample preparation is illustrated in Fig. 1.

3.3. Liberation of esterified steroids

The dry residue of the non-polar extraction was dissolved in 20 ml of the transesterification solution con-

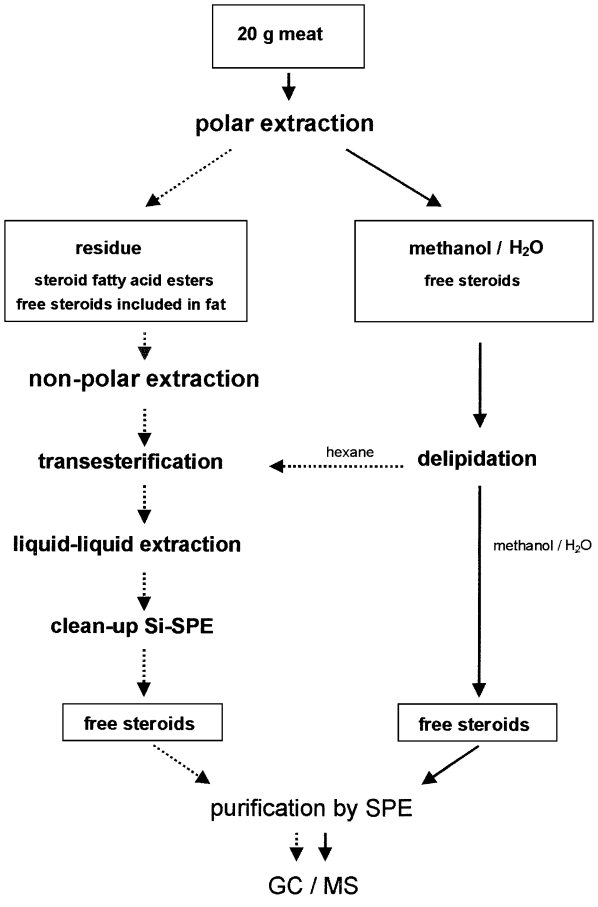

Fig. 1. Scheme of sample preparation.

sisting of 10% sodium methylate in methanol mixed with MTBE (4:6, v:v) by ultrasonication. After 30 min at room temperature the solution was neutralised with 2 M hydrochloric acid and extracted three times with 10 ml chloroform. The chloroform layers were collected and evaporated to dryness.

Samples were analysed with and without transesterification to determine steroid fatty acid esters. For analysis without transesterification the residue of non-polar extraction was dissolved in 20 ml methanol mixed with MTBE (4:6, v:v) and treated as described above. Contents of steroids with and without transesterification were compared. Higher contents in transesterificated samples indicate the occurrence of fatty acid esters.

3.4. Defatting of non-polar extracts by liquid–liquid extraction

The residue of the evaporated chloroform layer was dissolved in 70 ml methanol. Twenty millilitres of water were added. The solution was extracted twice with 20 ml *n*-hexane. Steroids were extracted three times from the methanol–water layer into 40 ml ethylene chloride. Ethylene chloride was evaporated by a vacuum evaporator (40 °C) and the residue dissolved in 200 μl chloroform and 2 ml *n*-hexane.

3.5. Clean up of steroids in non-polar extracts with Si-SPE

A Si-SPE cartridge was deactivated with 4 ml ethyl acetate containing 2.6% water. The conditioning was carried out with 4 ml *n*-hexane. The extract was applied to the cartridge. It was rinsed with 4 ml *n*-hexane, 4 ml *n*-hexane/MTBE (5:1, v/v) followed by 2 ml *n*-hexane/MTBE (3:1, v/v). Steroids were eluted with 6 ml ethyl acetate containing 2.6% water. The elute was given to 20 ml water and ethyl acetate was removed by a vacuum evaporator (40 °C).

3.6. Separation and clean up of the extract

Separation of steroids in corticoids, estrogens and other steroids and derivatisation to trimethylsilyl ethers and GC–MS analysis were carried out as described previously (Fritsche et al., 1999). The corticoid fraction was not analysed.

3.7. Determination of the intramuscular fat content

Beef (~30 g) was homogenised with a household mixer. One hundred millilitres of water and 150 ml 25% hydrochloric acid were added to 5–10 g of homogenised beef. The mixture was allowed to simmer for 45 min. Hot water (100 ml) was added and the hot mixture was filtered through a wet folded filter. The filter was washed with water until neutral reaction. The filter was dried and extracted with *n*-hexane by soxhlet extraction. Fat content was determined gravimetrically.

4. Results and discussion

4.1. Method

The investigated steroids were selected because of their biosynthetic relationship. They range widely in polarity due to different numbers of hydroxy- and keto-groups. Androstenedione is less polar because of missing hydroxy-groups. Pregnenolone and progesterone behave less polar because of the short side chain at C 17. The other investigated steroids react more polar. All standard substances of investigated steroids are soluble in methanol.

To consider polar steroids, non-polar steroids and steroid fatty acid esters, a methanol–water extraction combined with a non-polar extraction was chosen. As non-polar solvent *n*-hexane was chosen because standard substances of steroid fatty acid esters are soluble in *n*-hexane. Extraction of tissues with non-polar solvents after polar extraction is difficult because non-polar solvents are not able to penetrate tissues. To facilitate the *n*-hexane extraction the residue of polar extraction has to be pre-treated with a solvent with a polarity between methanol–water and *n*-hexane. Ethyl acetate was chosen because the polar extraction residues were penetrated by ethyl acetate. All steroids are soluble in ethyl acetate. Following the ethyl acetate extraction the residues were penetrated by *n*-hexane. The extraction procedure described here used solvents with a wide range of polarity. Consequently free steroids with different polarity, steroids embedded in cells with high fat content, and steroid fatty acid esters should be completely extracted.

The subsequent transesterification of the non-polar extract with sodium methylate solution allowed the analysis of steroid fatty acid esters as well as free steroids (Biedermann, Grob, & Mariani, 1993; Schmarr, Gross, & Shibamoto, 1996). Transesterification of steroid fatty acid esters resulted in free steroids and fatty acid methyl esters. Although saponification with methanolic potassium hydroxide solution also liberated steroids from fatty acid esters, this procedure was not recommendable. The resulting soapy residue showed emulsifying properties making a liquid–liquid extraction step impossible (Schmarr et al., 1996).

Also, the stability of corticoids was a problem during liberation of steroids from fatty acid esters. Our results show, that saponification as well as transesterification procedures destroyed the corticoid hormones. Probably, the corticoids were destroyed at the side chain at C17 because progesterone and pregnenolone, which also

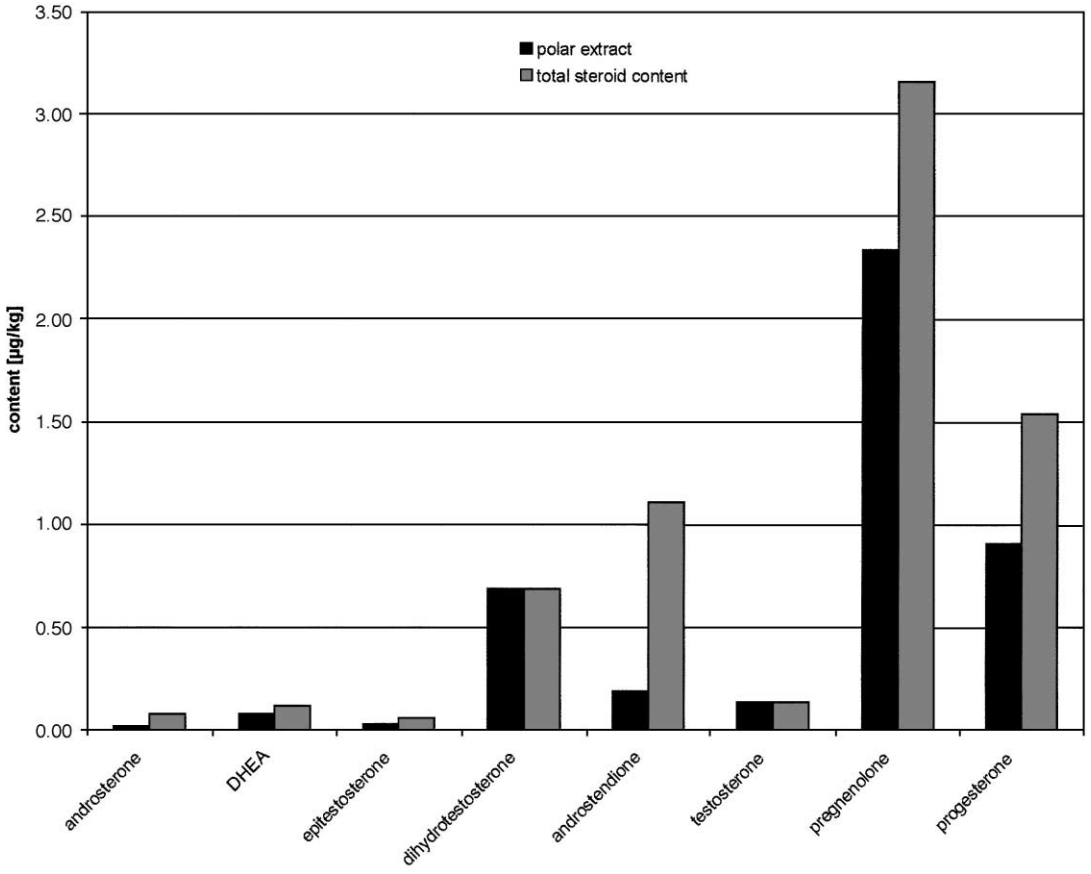

Fig. 2. Comparison of total steroid content and content of the polar extract in µg/kg beef of one sample.

have a side chain at C17, showed a similar behaviour at longer reaction times and higher temperature. The androgens resist these conditions. To minimise losses the transesterification procedure was performed at room temperature.

Extraction of steroids from the neutralised transesterification solution with chloroform included free and liberated steroids as well as fatty acid methyl esters. To remove compounds interfering with the chromatographic analysis the chloroform extract was evaporated to dryness and dissolved in methanol–water which was extracted with *n*-hexane. Steroids remained in the methanol–water layer if the ratio between methanol–water and *n*-hexane layer as well as extraction time were chosen as described. To remove remaining matrix compounds the extract was cleaned by Si-SPE and further purification was carried out as described previously (Fritsche et al., 1999). For the determination of steroid fatty acid esters each sample was also analysed without transesterification. An increased steroid content in transesterificated samples indicates the occurrence of steroid fatty acid esters.

4.2. Steroids in the non-polar extract

The steroid content of transesterificated extracts were not increased compared to non-transesterificated extracts. Consequently, no steroid fatty acid esters were detectable in analysed beef samples.

Steroid contents in non-polar extracts cannot be neglected as is shown in Table 1. Steroid contents of the non-polar extract are given as % of the total steroid content (sum of the contents of the polar and non polar extract). Total steroid contents are given in µg/kg.

Each investigated steroid is detectable in more than 5 of 8 investigated non-polar extracts. A reason for high deviations in amounts and patterns between the samples and undetectable amounts are individual differences of animals in steroid hormone formation and metabolism. The distribution of polar and less polar steroids between both extracts was different. More polar steroids are androsterone, DHEA, epitestosterone, dihydrotestosterone and testosterone, less polar are androstenedione, pregnenolone and progesterone. The more polar and physiological effective steroids testosterone and dihydrotestosterone showed highest contents in the non-polar extract contrary to the other steroids. In six of eight investigated samples testosterone was detected in the non-polar extract. In these cases, between 79 and

Table 1
Contents of steroids of the non-polar extract given as % of the total steroid content [*total steroid contents (μg/kg)*][a]

Steroid	Steer 1	Steer 2	Steer 3	Steer 4	Steer 5	Steer 6	Steer 7	Steer 8	*n*	Mean	Standard deviation
Androsterone	26 *(0.06)*	39 *(0.07)*	79 *(0.08)*	77 *(0.08)*	90 *(0.06)*	58 *(0.04)*	33 *(0.05)*	90 *(0.04)*	8	71	28
DHEA	70 *(0.08)*	56 *(0.10)*	32 *(0.12)*	80 *(0.15)*	100 *(0.06)*	0 *(0.03)*	47 *(0.14)*	67 *(0.15)*	8	57	31
Epitestosterone	n.n.	44 *(0.04)*	50 *(0.06)*	48 *(0.03)*	n.n.	n.n.	30 *(0.07)*	0 *(0.04)*	5	34	18
Dihydrotestosterone	n.n.	100 *(0.13)*	0 *(0.69)*	59 *(0.27)*	100 *(0.15)*	0 *(0.11)*	100 *(0.13)*	n.n.	6	45	47
Androstenedione	34 *(0.23)*	27 *(0.27)*	83 *(1.11)*	58 *(0.17)*	65 *(0.38)*	60 *(0.18)*	46 *(0.47)*	22 *(0.49)*	8	49	20
Testosterone	100 *(0.05)*	n.n.	0 *(0.14)*	89 *(0.90)*	100 *(0.11)*	88 *(0.08)*	79 *(0.12)*	83 *(0.12)*	7	77	32
Pregnenolone	37 *(1.40)*	41 *(1.71)*	26 *(3.16)*	36 *(0.81)*	32 *(3.17)*	48 *(0.99)*	41 *(1.16)*	38 *(1.11)*	8	38	6
Progesterone	85 *(0.51)*	31 *(2.40)*	41 *(1.54)*	60 *(0.60)*	34 *(1.80)*	49 *(0.48)*	45 *(1.22)*	44 *(0.51)*	8	51	16
Intramuscular fat content [g/100 g muscle tissue]	0.8	1.1	1.6	1.7	1.8	1.9	2.2	3.0	8	1.8	0.7

[a] *n*, number of values included in statistical analysis; DHEA, dehydroepiandrosterone; 0, detectable in the polar extract and not in the non-polar extract; n.n., not detectable in polar and non-polar extract.

100% of total testosterone were extracted with non-polar solvents. In one case, testosterone was not detectable in the non-polar extract and in one sample contents were below the determination limit in the polar as well as in the non-polar extract. If detectable dihydrotestosterone normally was completely in the polar or in the non-polar extract. Epitestosterone and androsterone are androgen metabolites. The epitestosterone content in the non-polar extract was less than 50%. Androsterone was detected in every non-polar extract and showed contents up to 90% in the non-polar extract.

The less polar steroids pregnenolone, progesterone and androstenedione were detected in every non-polar extract and ranged between 22–85% of the total content. The standard deviations were about 6% for pregnenolone, 16% for progesterone and 20% for androstenedione for the contents of steroids in the non-polar extract in relation to the total steroid content. Standard deviations of more polar steroids were higher than 18%. Total contents of progesterone, androstenedione and pregnenolone were higher than the contents of more polar steroids and ranged between 0.18 and 3.17 μg/kg. Polar steroids show contents up to 0.16 μg/kg except higher contents for dihydrotestosterone.

Steroid hormone patterns of polar extracts and of total steroids were comparable. An example of one beef sample is given in Fig. 2. All other samples behave in a similar way. The total steroid content is higher than the content of the polar extract. However, there were only small differences in proportions of single steroids.

The beef samples showed fat contents between 0.8 g/100 g muscle tissue and 3.0 g/100 g muscle tissue (Table 1). Neither the steroid content of the polar extract nor the steroid content of the non-polar extract seemed to be dependent on the intramuscular fat content. Consequently, the total steroid content was not dependent on the intramuscular fat content. The effect of individual differences between animals in hormone production seems to be higher than the effect of different fat contents. However, it should be mentioned that fat contents were generally low and comparable between all samples investigated.

Less polar steroids and androsterone were detectable in every non-polar extract contrary to other steroids. For this reason, extraction with polar solvents is insufficient for a complete extraction. The different contents of more polar steroids in the non-polar extract could be caused by the location of steroids in the tissue. Steroids placed in muscle cells with high fat content could not be extracted with methanol–water. A further reason is the good solubility of steroids in ethyl acetate.

Present work demonstrates that the content of steroid hormones in beef is higher than the content determined using only a methanol–water extraction. Polar extraction is not suitable for determination of steroids in cells with high fat content and less polar steroids. Steroid hormone patterns seem to be comparable for polar extraction and for the total steroid content. Steroid contents analysed by different extraction methods are not comparable. This result is relevant for a determination of anabolic treatment of animals, for example. Contents given by the EEC for an estimation of a treatment have to be considered from this angle. The determined amounts of steroids in beef and the estimated intake with beef containing food has to be reflected.

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