Project number: 871.639.01
BAS-code: WOT-02-438-III-036
Project title: Identification of unknown residues

Project leader: R.J.B. Peters

Report 2009.013 November 2009

# Identification of Unknown Residues

## using bioassay directed fractionation, UPLC/TOFMS analysis and database searching

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The research described in this report was funded by the Dutch Ministry of Agriculture, Nature and Food Quality (WOT-02-438-III-036)

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# Samenvatting


**Achtergrond**
Screeningsresultaten uit immunoassays, bioassays en microbiologische testplaten die niet bevestigd kunnen worden in de daaropvolgende chemische analyse werden tot voor kort als “vals-verdachte” screeningsresultaten terzijde gelegd. Sinds de ontdekking van illegale synthetische varianten, zoals de beta-agonist Clenbuterol-R en het anabole steroid THG in de sportdoping, bestaat de behoefte om in het kader van de opsporing van verboden middelen meer te doen aan de identificatie van bioactieve, mogelijk onbekende, (il)legale dierbehandelingsmiddelen. In surveys en quickscans is het wenselijk om in relatief korte tijd een zo breed mogelijk beeld te krijgen van residuen van dierbehandelingsmiddelen in bepaalde matrices. De introductie van UPLC/TOFMS en OrbitrapMS technologie biedt de mogelijkheid om grote groepen dierbehandelingsmiddelen in korte tijd chemisch te screenen.

**Doel**
Ontwikkeling en toepassing van massaspectrometrische identificatiemethoden voor nieuwe en bekende (il)legaal toegepaste dierbehandelingsmiddelen.

**Resultaten in 2008**
De effectgestuurde onderzoeksmethode bestaat uit een combinatie van een bioassay met een UPLC/TOFMS instrumentele analyse. Het onderzoek is uitgevoerd aan 14 zogenaamde “cold-cases”, oude monsters voedersupplementen en –preparaten, waarin destijds geen relevante stoffen konden worden aangetoond ondanks het feit dat er volgens de informatie van de AID sprake was van bioactieve werkzaamheid, en aan acht monsters kruidenpreparaten en sportsupplementen die mogelijk stimulerende bioactieve stoffen bevatten en in de loop van 2008 zijn verzameld. De monsters zijn in eerste instantie onderzocht met behulp van een experimentele bioassay die bestaat uit gemodificeerde gistcellen die na blootstelling aan androgene stoffen fluorescentie vertonen. Er is tevens een aantal specifieke (bio)activeringen toegepast om naast de directe androgenen ook pro-androgenen, androgeen-esters en geconjugeerde androgenen en pro-androgenen te kunnen bepalen. Uit de resultaten blijkt dat in geen van de “cold cases” androgene activiteit wordt gedetecteerd, ook niet na specifieke activering. Hieruit wordt geconcludeerd dat deze monsters geen androgenen, pro-androgen, androgeen-esters of geconjugeerde androgenen of pro-androgenen bevatten. Twee van de vier kruidenpreparaten bleken positief te zijn in de bioassay, terwijl drie van de vier sport supplementen "indicatief" of toxisch reageerden in de bioassay. Een indicatieve respons wijst op de aanwezigheid van androgeen-achtige stoffen en kan aanleiding zijn tot verder onderzoek. De positieve kruidenmonsters zijn gefractioneerd met LC, de fracties opnieuw op bioactiviteit getest en de positieve fracties geanalyseerd met UPLC/TOFMS. In de positieve fractie van één monster werden testosteronphenylacetaat (of het structurele isomere nortestosteronphenylpropionaat) en testosteronhexahydrobenzoaat aangetroffen. In het tweede monster werd een methyltestosteron isomeer geïdentificeerd. Door een onafhankelijke analyse met chemische standaarden konden de identiteiten worden bevestigd als nortestosteronphenylpropionaat en 17α-methyltestosteron die in de monsters aanwezig waren in geschatte concentraties van respectievelijk 0,2 en 4 mg/kg product.

In het kader van dit project, de identificatie van onbekende stoffen, is voor de UPLC/TOFMS analyse een accurate massa database ontwikkeld op basis van de PubChem database die op het internet kan worden gevonden. Dit heeft in eerste instantie geleid tot een off-line versie in Windows Access format en voorzien van een gebruikersinterface waarin de zoekcriteria zoals accurate massa en massa window kunnen worden opgegeven. Daarnaast is de accurate massa database ook gekoppeld met de TOFMS software om on-line onbekende componenten te kunnen identificeren in de sportsupplementen die indicatief en toxisch reageerden in de bioassay. Door de UPLC/TOFMS data files vooraf te bewerken met MetAlign (in-huis ontwikkelde software voor het opschonen van MS data) en deze files verder te processen was het mogelijk onbekende componenten in de chromatogrammen van de extracten van de sportsupplementen te identificeren. Dit leidde in drie sportsupplementen tot de identificatie van de androgenen methylboldenon en testosteron, en de androgeen esters methyltestosteronpropionaat (of het isomere testosteronisobutyraat), testosteronbuciclaat en methyleentestosteronacetaat. Daarnaast werden in deze monsters een aantal norcodeine- of morphine-achtige componenten geïdentificeerd.

**Conclusie**

In de “cold cases” zijn geen stoffen met androgene werking aangetoond. Bioassay gestuurde fractionering gecombineerd met UPLC/TOFMS analyse blijkt in staat stoffen met androgene werking te kunnen detecteren, isoleren en identificeren. In twee kruidenmengsels die als sport supplementen worden toegepast zijn zo androgenen en respectievelijk androgeen esters gedetecteerd en na analyse met UPLC/TOFMS geïdentificeerd. Daarnaast blijkt de combinatie van UPLC/TOFMS analyse met een accurate massa database goed in staat onbekende componenten te identificeren. Met deze methode werden in dit onderzoek meerdere androgenen en androgeen esters in drie sportsupplementen geïdentificeerd. Het doel van dit onderzoek is hiermee gehaald.

# Summary


**Background**
Nowadays a large number of compounds are determined in environmental and food samples. Biological tests are used to screen samples for large groups of compounds having a particular effect, but it is often difficult to identify a specific compound when a positive effect is observed. The identification of an unknown compound is a challenge for analytical chemistry in environmental analysis, food analysis, as well as in clinical and forensic toxicology. This study reports on the development of a procedure for the identification of unknown residues in samples suspected of containing illegal substances and samples showing bioactivity in bioassay- or microbiological screening assays. For testing purposes several samples were selected; a number of so-called “cold cases”, historical samples that were suspected of containing illegal growth promoting substances, herbal mixtures and sport supplements.

**Aims**
The development of an identification procedure for unknown biological active compounds using liquid chromatography in combination with time-of-flight mass spectrometry.

**Results**
In this study bioassay directed fractionation combined with UPLC/TOFMS identification was tested as a method to detect and identify unknown androgens. Test samples comprised of a series of “cold cases”, feed supplements suspected to contain growth promoting substances already analyzed in the past, however, without detecting any such compounds. A second set of samples consisted of herbal mixtures and sport supplements suspected to contain compounds with androgenic activity. The results of the androgen bioassay tests on “cold cases” showed that none of the “cold-case” samples contained compounds with androgenic activity. Not only the direct androgen bioassay showed no response, but also the tests for pro-androgens, androgen esters and conjugated androgens and pro-androgens were negative indicating that no compounds are present that can be metabolically converted into androgens. Two of the four herbal mixtures tested positive, one for the presence of an androgen, another for an androgen ester. Bioassay guided fractionation of the positive herbal mixtures resulted in the fractions of the sample chromatogram where the androgenic compounds elute. Further analysis of these fractions with UPLC/TOFMS resulted in the tentative identification of a methyltestosterone in one of the samples and testosterone phenylacetate (or the isomeric nortestosterone phenylpropionate) and testosterone hexahydrobenzoate in another. Using a confirmation method the methyltestosterone isomer in the herbal mixture was standard confirmed as 17α-methyltestosterone present in a concentration of approximately 4 mg/kg product. The identity of the androgen ester was standard confirmed as nortestosteron phenylpropionate with a concentration of 0.2 mg/kg product in the herbal mixture. An accurate mass database containing approximately 40,000 compounds was developed based on the PubChem database on the internet. At first this database was developed into an off-line custom database in Windows Access format with a user interface for entering search parameters and a link to the original PubChem database. The database was also coupled to the TOFMS software allowing reversed searching and automatic identification of chromatographic peaks without primary reference standards. The combination of UPLC/TOFMS with data processing and accurate mass database searching was used to analyze the sport supplements since these were negative, inconclusive

or reacted toxic in bioassay tests. Without the use of primary reference standards the analysis resulted in the identification of several androgens, including methylboldenone, testosterone and the androgen esters methyltestosterone propionate or testosterone isobutyrate, testosterone buciclate and methylenetestosterone acetate in the sport supplements. In addition, a number of norcodeine- or morphine-like compounds were found in these samples.

**Conclusion**
The “cold-case” samples do not contain any androgens, pro-androgens, androgen esters or conjugated androgens. The study showed that bioassay guided fractionation in combination with UPLC/TOFMS analysis is a successful procedure to detect and identify unknown androgens in herbal mixtures and sport supplements. The use of large databases without primary standards and coupled with the instrument software enables the automatic identification of peaks in the chromatogram and looks very promising for unknown identification. The aims of the study have been met.

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# 1 Introduction

Nowadays a large number of compounds are determined in environmental and food samples. In almost all cases this concerns substances that are already known and the general concern is to confirm their presence and to determine the concentrations of these substances. However, many more substances for which no specific tests are performed may be present in the sample. Biological tests can be used to screen samples for large groups of compounds having a particular effect, but it is often difficult to identify a specific compound when a positive effect is observed. Similar problems are encountered in toxicological analysis, for instance in cases of acute poisoning. The identification of an unknown compound is a challenge for analytical chemistry in environmental analysis, food analysis, as well as in clinical and forensic toxicology. The screening of unknown compounds is also called “General Unknown Screening” (GUS) or sometimes “Systematic Toxicological Analysis” (STA) (De Zeeuw 1997; Sturm 2005). The purpose of such methods is to identify (xenobiotic) substances in environmental, food and biological matrices. Following the usual course of STA, samples will initially be analyzed by immuno- or bioassays (Ferrara et al. 1994; Nielen et al. 2003 and 2004; Hino et al. 2003). These preliminary biological screening procedures mainly concern rapid-response analytical tools providing a binary “yes/no” response, which indicates whether the target analytes are present above a preset concentration threshold or not. Samples providing a “yes“ response to one or more compound classes or target substances are than analyzed with a confirmation method.

Analysis of veterinary drugs, hormones, pesticides and their metabolites in food and biological samples is an important routine task for food safety, doping control and clinical and forensic toxicology laboratories. Monitoring of these compounds is a particularly demanding task for both analytical and interpretive reasons, because of the extremely wide range of substances, in terms of molecular weight, polarity, pKa, and chemical/thermal stability. As a consequence, recently several examples of broad screening methods for known compounds in environmental, food, feedstuff, toxicological and biological samples have appeared (Lacorte et al. 2006; Marques et al. 2006; Portolés et al. 2007; Kolmonen et al. 2007, Kaufmann et al. 2007; Mol et al. 2008; Kaufmann et al. 2008; Stolker et al. 2008; Peters et al. 2009). Even more than these methods, GUS and STA procedures require the primary extraction to be generic and non-discriminative against relevant compounds. Purification methods like SPE can be used to remove non-relevant substances and matrix interferences, but should be used with caution since they will compromise the generic nature of the sample preparation. An exception can be made if the unknown compound belongs to a certain class of compounds, for instance estrogens, β2-agonists or antibiotics, and the bioassay is able to differentiate between these compound classes. This would allow a compound-class directed clean-up procedure to remove non-relevant compounds. The next step in GUS is a separation of the analytes, often using chromatography.

While many methods have been used in the last few years, it is safe to say that liquid chromatography (LC) is the most generic of these, and since the introduction of high resolution LC (HRLC) also one of the most selective. HRLC, ultra performance LC (UPLC) and ultra high performance LC (UHPLC) are all synonyms for LC separations on sub-2-μm particulate packing materials which provides significant advantages concerning selectivity, sensitivity and speed. Gas chromatography (GC) has been used but is limited to apolar, volatile and thermally stable compounds (Maurer 2004). Finally, the

detection should be generic and sensitive, allowing the identification of compounds using databases or reference substances. Coupling of mass spectrometry (MS) to LC seems to be the possibility to increase the range of compounds amenable to MS (Marquet 2002). Especially full mass scan MS techniques like time-of-flight MS (TOFMS) or OrbitrapMS provide high specificity due to both, high mass accuracy and high mass resolution and allow the reconstruction of highly selective accurate mass chromatograms of target residues in complex matrices. The advantage of TOFMS and OrbitrapMS analyzers is their ability to analyze a sample for a theoretically unlimited number of compounds and therefore, the combination of these analyzers with LC is capable of screening for several hundreds of compounds with high sensitivity within one run. Furthermore, data can be acquired and reprocessed without any a priori knowledge about the presence of certain compounds; that is, no analyte-specific information is required before injecting a sample and the presence of newly identified compounds can be confirmed in previously analyzed samples simply by reprocessing the data. The advantage of these analyzers can be further improved by combining it with HRLC. In the screening of veterinary drugs HRLC-TOFMS has been shown to provide significant advantages concerning selectivity, sensitivity and speed (Kaufmann et al. 2007 and 2008; Mol et al. 2008; Stolker et al. 2008; Peters et al. 2009). For HRLC-OrbitrapMS these advantages are even higher, because of the higher selectivity and sensitivity of the OrbitrapMS when compared to TOFMS (Van der Heeft et al. 2009).

The use of HRLC/TOFMS generates an enormous amount of data potentially allowing the identification of unknown compounds. However, depending on the matrix, the enormous amount of data can also cause problems in identification, because screening may be hampered by matrix interferences and manual searching of TOFMS data for unknown compounds is difficult. The large number of peaks in a chromatogram may result in a “forest of peaks” making it difficult to distinguish between true compounds of interest or “active” compounds, and compounds resulting from the matrix or endogenously compounds from biological matrices. One way to pinpoint the bioactive unknown compounds is generally known as bioassay directed fractionation. The sample extract is then fractionated with the help of LC and the eluent split in a dual collection system with 96-well micro-titre plates. Collection is usually carried out as a function of time and a fraction volume of up to 300 μL is used depending on the type of bioassay (Dittmann et al. 2004; Vuorela et al. 2004; Waridel et al. 2004; Queiroz et al. 2005). The LC-MS data from the original sample extract or the separate fractions can be matched and correlated with the bioactivity profile from the separate fractions to identify peaks of bioactive compounds in the chromatogram. Finally, this information can be used to search compound databases for the tentative identification of the bioactive unknown compounds. While this procedure is primarily used in the identification of natural products (Hamburger 2003; Wennberg 2006; Han et al. 2009) it has also been used in the analysis of steroids in surface water (Beck et al. 2006) and urine (Nielen et al. 2004 and 2006), and veterinary drug residues in food and feed (Marchesini et al. 2007). This methodology is illustrated in Figure 1.

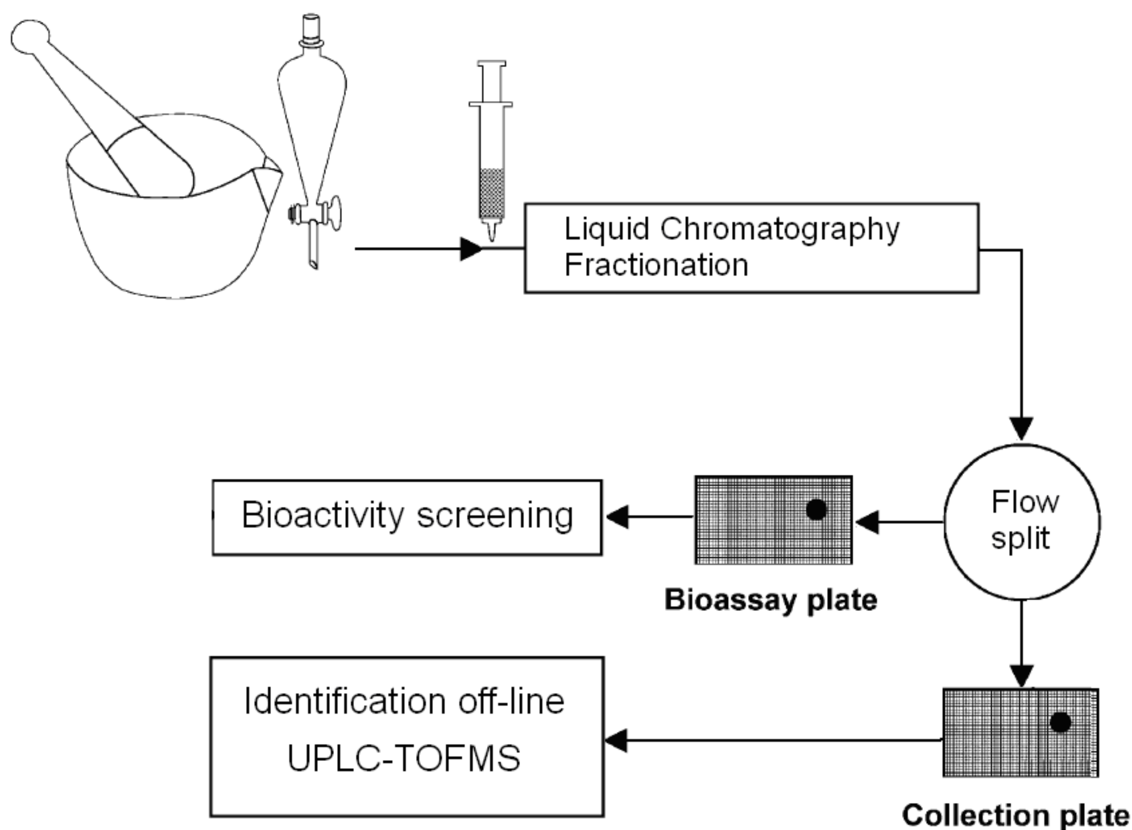


*Figure 1. Schematic presentation of bioassay guided fractionation and identification of unknown bioactive compounds in bioactive fractions using UPLC-TOFMS.*

While LC/MS is a more generic detection technique than GC/MS it also has a disadvantage. Different from GC/MS, LC/MS techniques generally do not produce interpretable full mass spectra, firstly since the soft ionization techniques used show little or no fragmentation, secondly because techniques like in-source collision-induced dissociation (CID) show spectra with poor reproducibility, especially when acquired with instruments from different manufacturers. As a consequence, searchable databases as the well known National Institute for Standards and Testing (NIST) mass spectral library for GC/MS are virtually absent for LC/MS (NIST 2008). It is the unavailability of such mass databases that doesn't allow a rapid screening of samples with LC/MS. In contrast, accurate mass measurements like those performed by high resolution TOFMS are specific and universal for every compound regardless of the instrumentation used, potentially enabling the use of mass databases. Thurman used this approach, a combination of TOF data and the Merck Index to identify pesticides in food, including degradation products, without the initial use of primary standards (Thurman et al. 2005; Ferrer et al. 2006). Another interesting approach was used by Laks who determined street drugs without primary reference materials. Identification was performed by LC/TOFMS essentially based on accurate mass determination using a target database of 735 exact monoisotopic masses (Laks et al. 2004). To prepare the database theoretical monoisotopic exact masses of compounds were calculated from their molecular formula. Peaks in a chromatogram were identified using a mass window criterion of ±20 ppm for compounds of >200 Da and ±30 ppm for compounds <200 Da. In addition a minimum area count was used and a retention window of ±0.2 min if a retention time was available. A similar procedure was used for the screening of drug residues but included isotopic pattern matching as an orthogonal criterion for compound identification (Ojanperä et al. 2006). Of course, without primary

reference standards identifications have only a tentative character and the identity should be confirmed using the actual standard compounds in a suitable confirmation method.

This study reports on the development of a procedure for the identification of unknown residues in samples suspected of containing illegal substances and samples showing bioactivity in immunoassays, bioassays or other microbiological screening assays. For testing purposes a number of so-called “cold cases” were selected, historical samples that were suspected of containing illegal substances (in particular hormones), but whose presence was never confirmed with bioassays or chemical analysis. In addition, a number of herbal preparations and sport supplements, also called ergogenic aids, were tested. The development of the identification procedure contains the application of an existing bioassay directed identification procedure in combination with advanced HRLC/TOFMS techniques and the development of a database for compound identification in LC/TOFMS analysis.

# 2 Methods

## 2.1 Chemicals and reagents

All solvents were of HPLC-grade or higher. Acetonitrile, methanol acetone, isooctane and water used for LC/MS measurements were purchased from Biosolve (Valkenswaard, The Netherlands). Water used for sample preparations was purified using a Millipore Milli-Q system (Bedford, MA, USA). Sodium acetate, sodium (bi)carbonate and leucine-enkephalin were purchased from Sigma (Zwijndrecht, The Netherlands). Ethyl acetate, formic acid, acetic acid, sodium acetate, sodium carbonate, sodium chloride, di-sodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate, ammonium sulphate, magnesium chloride, potassium chloride, tris(hydroxymethyl)aminomethane (Tris), dimethylsulfoxide (DMSO), hydrochloric acid and acetic acid were purchased from Merck (Darmstadt, Germany). Glucose-6-phosphate, NADH disodium salt, NADP disodium salt and NADPH tetrasodium salt were from Roche Diagnostics (Almere, the Netherlands). Dextrose and yeast nitrogen base without amino acids and without ammonium sulphate were from Difco (Detroit, MI, USA). L-Leucine, bovine serum albumin (BSA), hydroxysteroid dehydrogenase originating from Pseudomonas, testosterone and NAD sodium salt were purchased from Sigma (St. Louis, MO, USA).

## 2.2 Samples

Two different types of samples were involved in this study. The first group consisted of historical samples received from the AID because they suspected these samples to contain illegal substances. All samples were received in the period from 2005 to 2008. At the time of their receipt the samples were generally analyzed using NMR, a number of targeted chemical analyses, and sometimes a chloramphenicol immunoassay and an estrogen bioassay. The samples that were used in this study and the results of their analysis at that time are as follows:

| RIKILT-code | Sample description | Results of prior analysis |
|---|---|---|
| 200141597 | feed | negative |
| 200141598 | feed | negative |
| 200144635 | feed bigbag | positive for estrogens |
| 200144690 | supplement for hypophysis activity | negative |
| 200145516 | cod-liver oil | negative |
| 200145518 | herbal mixture | negative |
| 200146583 | herbal mixture | positive for dexamethasone |
| 200146585 | herbal mixture | positive for dexamethasone |
| 200146589 | herbal mixture | positive for dexamethasone |
| 200148736 | all mash | negative |

| RIKILT-code | Sample description | Results of prior analysis |
|---|---|---|
| 200166957 | feed | negative |
| 200207042 | herbal mixture | positive for chloramphenicol |
| 200207177 | digestamin piglet | positive for estrogens |
| 200207178 | digestamin fattening | negative |

In addition, a number of sport supplements were used in this study. These samples were received from sport schools or intermediates. The supplements are the following:

| Name | Sample description | Intended use |
|---|---|---|
| Herb A1 | brownish herbs in brown capsule | chinese herbs for improved performance in sports |
| Herb A2 | brownish herbs in brown capsule | chinese herbs for improved performance in sports |
| Herb A3 | reddish herbs in orange capsule | indonesian herbs for improved performance in sports |
| Herb A4 | reddish herbs in orange capsule | indonesian herbs for improved performance in sports |
| Suppl. S1 | white powder in capsule | sports supplement |
| Suppl. S2 | white powder in capsule | sports supplement |
| Suppl. S3 | white powder in capsule | sports supplement |
| Suppl. S4 | white powder in capsule | sports supplement |

## 2.3 Sample analyses

### *2.3.1 Sample preparation for androgen bioassay*

The sample preparation method is comparable to some of the recent published multi-methods. A sample of 3 gram is mixed with 6 ml methanol and 6 ml sodium acetate buffer (0.25M; pH 4.8) and shaken by hand. Next, it is placed 10 min in an ultrasonic bath followed by 15 min in a head-over-head apparatus. Finally, the mixture is centrifuged for 15 min at 3500 g and 6 ml of the supernatant is collected in a glass tube. To this 150 µl acetic acid (4.0 M) is added and the pH checked to be about 4.8. For the first SPE purification a Varian $C_{18}$ cartridge (1000 mg, 6ml) is conditioned with 4 ml methanol followed by 3 ml of a 1:1 mixture of methanol and sodium acetate buffer (0.25M; pH 4.8) before applying the 6 ml extract to the cartridge. The cartridge is washed in succession with 3 ml of a 1:1 mixture of methanol and sodium acetate buffer (0.25M; pH 4.8), 6 ml milli-Q water, 3 ml of a sodium carbonate solution (0.25M), 3 times 6 ml milli-Q water and 2 times 4 ml of a 1:1 mixture of

methanol and milli-Q water. The cartridge is dried for 10 min by applying vacuum and eluted with 2 times 4 ml acetonitrile. Again, the eluates are collected in a glass tube to avoid contamination from plastic materials. For the second SPE purification an Isolute $NH_2$ cartridge is activated with 4 ml of acetonitrile. The 8 ml eluate of the first SPE is brought on top of the cartridge and the eluate is collected in a glass tube. Finally, the eluate is evaporated to dryness under a stream of nitrogen gas in a TurboVap apparatus at 40°C and the residue reconstituted in 3 ml of acetonitrile.

### *2.3.2 Sample pre-treatment for glycosides (enzymatic digestion)*

In order to remove glycoside groups from androgens an enzymatic digestion of the sample is included as a pre-treatment. Typically, a sample of 100 mg is mixed with 4 ml sodium acetate buffer (0.25M; pH 4.8) and shaken by hand. Next, 110 µl glucuronidase reagent is added for an enzymatic deconjugation for 3 h at 52°C. After cooling to room temperature 4 ml methanol is added, the mixture vortexed and shaken for 10 min in a head-over-head apparatus. The mixture is centrifuged for 15 min at 3500 g and 4 ml of the supernatant is collected in a glass tube. The pH is checked and if necessary corrected to 4.8 by the addition of acetic acid (4.0 M). An SPE purification is applied using an Oasis HLB cartridge (60 mg, 3ml) conditioned with 2 ml methanol followed by 2 ml of milli-Q water before applying the 4 ml extract to the cartridge. The cartridge is washed in succession with 2 ml of milli-Q water, 2 ml of a 1:1 mixture of methanol and milli-Q water. The cartridge is dried for 10 min by applying vacuum and eluted with 4 ml methanol.

### *2.3.3 Sample preparation for pro-hormones (enzymatic activation)*

Pro-hormones in the sample can be detected only after activation and therefore an enzymatic activation using an S9 mixture is applied as a pre-treatment. Typically, 100 µl of the final SPE eluent from samples (see "Sample preparation for androgen bioassay") was evaporated to dryness in a glass tube and the residue incubated with 500 µl sodium phosphate buffer (0.2M; pH 7.4), 50 µl glucose-6-phosphate (0.2M), 20 µl potassium chloride solution (1.65M), 20 µl magnesium chloride solution (0.4M), 100 µl bovine S9 mixture (20 mg/ml in a Tris-HCl buffer of 50 mM, pH 7.4, 1.15% KCl) and 40 µl NAD or NADPH (0.1M). The final volume was made up to 1.0 ml using milli-Q water, the tube closed and incubated at 37°C in a water bath for 6 h. Blanks without bovine liver S9 and blanks without cofactor were included to check for non-enzymatic reactions during the incubation period. Reactions were terminated at t=0 and t=6 h by the addition of 1 ml acetonitrile and the mixture centrifuged for 15 min at 3000 g. The supernatant was collected, the residue extracted once more with 2 ml acetonitrile and centrifuged for 10 min at 3000 g. The combined supernatants were evaporated to appr. 0.5 ml and diluted with 3 ml methanol, centrifuged for 15 min at 3000 g and evaporated until dryness. The residue was dissolved in 200 µl methanol and diluted to 2 ml with milli-Q water. Finally, a SPE purification is applied using an Oasis HLB cartridge (60 mg, 3 ml) conditioned with 2 ml methanol followed by 2 ml of milli-Q water before applying the 2 ml extract to the cartridge. The cartridge is washed twice with 2 ml of milli-Q water and eluted with 2 ml methanol.

### *2.3.4 Sample preparation for steroid esters (enzymatic de-esterification)*

Enzymatic hydrolysis of the steroid esters was carried out using esterase from porcine liver. Typically, 100 µl of the final SPE eluent from samples (see "Sample preparation for androgen bioassay") was evaporated to dryness in a glass tube and the residue incubated with 500 µl sodium phosphate buffer

(0.2M; pH 7.4), 100 µl esterase (20 mg/ml in a Tris-HCl buffer of 50 mM, pH 7.4, 1.15% KCl). The final volume was made up to 1.0 ml using milli-Q water, the tube closed and incubated at 37°C in a water bath for 6 h. After cooling to room temperature 1 ml acetonitrile was added and the mixture centrifuged for 15 min at 3000 g. The supernatant was collected, the residue extracted once more with 2 ml acetonitrile and centrifuged for 10 min at 3000 g. The combined supernatants were evaporated to approximately 0.5 ml and diluted with 3 ml methanol, centrifuged for 15 min at 3000 g and evaporated until dryness. The residue was dissolved in 200 µl methanol and diluted to 2 ml with milli-Q water. Finally, a SPE purification is applied using an Oasis HLB cartridge (60 mg, 3 ml) conditioned with 2 ml methanol followed by 2 ml of milli-Q water before applying the 2 ml extract to the cartridge. The cartridge is washed twice with 2 ml of milli-Q water and eluted with 2 ml methanol.

### *2.3.5 Fractionation of extracts*

The fractionation system consisted of two Knauer (Berlin, Germany) model WellChrom K-1001 pumps, a Knauer high-pressure dynamic mixing chamber and a Separations (H.I.Ambacht, The Netherlands) model 920 autosampler. Liquid chromatography was performed using a Waters (Milford, MA) 150 x 3.0 mm i.d. Symmetry column packed with 5 µm C18 packing and a mobile phase consisting of (A) water/acetonitrile (90:10) and (B) water/acetonitrile (10:90). Gradient elution was performed at a flow rate of 0.4 ml/min, starting at 35% B and linearly programmed to 100% B in 20 min. The column effluent was split toward two Gilson (Villies-le-Bel, France) model FC203B 96-well fraction collectors.

### *2.3.6 Recombinant yeast androgen assay*

Aliquots of 200 µl of final extracts were pipetted in a conical 96-well plate (Greiner Bio-One, Germany) and 50µl of a 4% solution of DMSO in milli-Q water was added. The plate was left overnight in a fume cupboard to allow the acetonitrile to evaporate and leave only the DMSO the next day. Aliquots of 200 µl yeast suspension were added to each well of the 96-well plate. A standard dose-response curve of 17β-testosterone was included in each experiment. Plates were incubated at 30°C for 24 h in a shaking incubator (125 rpm), and fluorescence was measured (485 nm excitation, 530 nm emission) using a SynergyTM HT multidetection microplate reader (BioTek Instruments Inc., USA). The optical density (OD) of the yeast was measured at 630 nm after 24 h to monitor for any cytotoxic effects on the yeast cells.

### *2.3.7 Instrumental analysis: Chromatographic conditions*

Separation of the sample was performed on a Waters Acquity UPLC system consisting of a vacuum degasser, an autosampler with a cooled sample tray, a column oven and a binary solvent manager with high pressure mixing chamber (Waters, Milford, MA, USA). Elution was performed at a stable temperature of 35 ºC using a Waters Acquity BEH-$C_{18}$ column (100 x 2.1 mm i.d., 1.7 µm particle size). The eluents consisted of 0.1% formic acid (A) and acetonitrile/0.1% formic acid in water, 9/1 (v/v) (B). Ultra pure, LC/MS quality water was used to eliminate excessive background signals and avoid the formation of sodium or potassium adducts. A step-wise gradient starting at 0% B was employed at a flow of 0.4 ml/min. From 1 to 4 min the %B was linearly increased to 40% and during 4 to 10 min linearly increased to 100% with a final hold for 2 min. The total run-to-run time (including equilibration prior to injection of the next sample) was 13 min. The injection volume was 20 µl.

### 2.3.8 *Instrumental analysis: Mass spectrometry conditions*

The effluent of the HRLC system was directly interfaced to a Bruker Daltonics micrOTOF mass spectrometer equipped with an orthogonal electrospray ionisation (ESI) source, operated in the positive mode using a mass range of 100 to 1000 Da. The trigger time was 33 µs and 10,000 spectra were summed up equaling 0.33 s time resolution. The capillary voltage of the ion source was set at 3500 V and the capillary exit at 100V. The nebulizer gas pressure was 1.5 L/min and drying gas flow 8 L/min. The drying temperature was set at 200 °C. Instrument calibration was performed externally prior to each sequence with a sodium formate/acetate solution, consisting of 3.3 mM sodium hydroxide in a mixture of water/isopropanol/formic acid/acetic acid (1:1:1:3, v/v). The theoretical exact masses of calibration ions with formula $Na(HCO_2Na)_{2\text{-}8}$ and $Na(CH_3CO_2Na)_{2\text{-}8}(HCO_2Na)_{2\text{-}8}$ in the range of 100 to 1000 Da were used for calibration. Automated post-run internal mass scale calibration of individual samples was performed by injecting the calibrant at the beginning and the end of each run via a six-port divert valve equipped with a 20 µL loop. The actual calibration was performed based on calibrant injection at the beginning of the run while the calibrant at the end of the run was for manual verification of calibration stability. The calibrator ions in the post-run internal mass scale calibration were the same as in the instrument calibration.

### 2.3.9 *Data base construction*

PubChem is a database of chemical molecules (PubChem 2009). The system is maintained by the National Center for Biotechnology Information (NCBI), a component of the National Library of Medicine, which is part of the United States National Institutes of Health (NIH). PubChem contains substance descriptions and small molecules with fewer than 1000 atoms and 1000 bonds and can be accessed freely through a web user interface. This option is used to set up a large accurate mass database from the PubChem database. It should be mentioned that at the same time the construction of a similar database was reported by Polettini who used this in combination with capillary electrophoresis ESI/TOFMS (Polettini et al. 2008). A different approach was used by Thurman who developed an identification scheme for unknown pesticides using TOFMS and searching for the empirical formula using the accurate mass and the Merck Index database (Thurman et al. 2005). The PubChem data files were downloaded as text files and converted to an Excel format limiting the database to a maximum of 65,000 compounds. These text files contain the trivial and IUPAC name of the compounds, the molecular formula and some other information including a PubChem identification number. Unfortunately, the database does not contain any CAS numbers which would have made the identity and exchangeability with other databases much easier.
At this point the database contains many compounds that are not suitable for MS detection, not of interest for our purpose, or in an ionic form that is not relevant for UPLC-TOFMS analyses. In order to remove as many as possible of these compounds and to reduce the size of the database, a subset was created by applying the following limitations: a, Molecular mass range from 100 to 750: b, Elements limited to C, H, O, N, F, Cl, Br, I, P, S: c, Double entries (several identical compounds with different trivial names are included) were removed as far as possible. The final database contains approximately 40,000 compounds. The theoretical exact mass of the protonated compounds was calculated from the elemental compositions using exact atomic masses and correcting for the loss of an electron (i.e. calculated as $[M+H]^+$). The database was sorted into columns in the order of exact masses, elemental compositions, trivial and IUPAC names, and PubChem identification number. The format was converted to make the database compatible with the instrument data analyses software Target Analysis™ (Bruker) and Search LC™ (Thermo Analytical). In addition, the Excel table was converted

to a Windows Access format, and a custom database with a user interface was generated in which different parameters such as the type of TOF instrument (Waters LCT versus Bruker TOF) being used, the mode of ionisation (positive versus negative), the accurate mass and allowed search mass window can be entered. In addition, for each compound the database contains a link to the original PubChem database on the internet to find additional information. Figure 2 gives a picture of the user interface of the Access format database.

Component search by Mass Approach

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Component search by Mass Approach

Show results for: Bruker TOF / Waters LCT

Component search: Pos. mode (M+H) / Neg. mode (M-H) — Mass: 311.1206 — Mass window: 0.005 — Search — Report

| Exact Mass (M+H) Bruker TOF | Abundance M+2 | Abundance M+3 | Exact Mass (M) | Trivial Name | Molecular Formula | PubChem Code |
|---|---|---|---|---|---|---|
| 311.115691 | 18 | 34 | 310.108421 | Ro 03-7894; 1-(5-Chloroacetylaminobenzofuran-2-yl)-2-isopropylaminoethanol; Acetamide, 2-chloro-N-(2-(1-hydroxy-2-((1-methylethyl)amino)ethyl)-5-benzofuranyl)- | C15H19ClN2O3 | 194324 |
| 311.117232 | 17 | 6 | 310.109962 | 2-Aatsc; N(4)-(2-Acetoxyethoxymethyl)-2-acetylpyridine thiosemicarbazone; Hydrazinecarbothioamide, N-((2-(acetyloxy)ethoxy)methyl)-2-(1-(2-pyridinyl)ethylidene)- | C13H18N4O3S | 9576879 |
| 311.123758 | 17 | 2 | 310.116488 | gamma-Glutamyltyrosine; N-L-gamma-Glutamyl-L-tyrosine; EINECS 231-076-5 | C14H18N2O6 | 94340 |
| 311.123758 | 17 | 2 | 310.116488 | para-Ethoxy-ida; p-Ethoxy-ida; 4-Ethoxy-ida | C14H18N2O6 | 191673 |
| 311.123758 | 17 | 2 | 310.116488 | EINECS 219-621-5; N-tert-Butyloxycarbonylalanine-4-nitrophenyl ester; 4-Nitrophenyl N-((1,1-dimethylethoxy)carbonyl)-L-alaninate | C14H18N2O6 | 102826 |
| 311.124443 | 12 | 6 | 310.117173 | EMGBG sulfate; EMGBG; NSC614860 | C7H18N8O4S | 9569752 |

Record: 1 of 8 (Filtered)

*Figure 2. User interface of the off-line accurate mass database that was constructed in Windows Access format for the identification of compounds based on their accurate mass.*

# 3 Results and Discussion

## 3.1 Primary detection of unknown, biological active substances

The detection and identification of growth promoting, androgenic, compounds was taken as the subject for this study. The use of growth-promoting compounds in cattle fattening is prohibited in the European Union according to EC directive 96/22 (EC 1996). The EC 96/22 does not contain a list of specific compounds that are prohibited, but instead states that all substances having thyrostatic, estrogenic, androgenic or gestagenic activity are prohibited. In the same way steroids that enhance the performance in sports are on the list of prohibited substances of the World Anti-Doping Agency (WADA) (WADA 2008). In livestock production as well as in sports a trend has been observed from the abuse of synthetic steroids towards natural steroid esters, pro-hormones and designer modifications of existing drugs and the recently discovered “designer” modifications are representative of this trend (Pearce et al 2006; Catlin et al. 2002; Catlin et al. 2004; Parr et al. 2009). Pro-hormones such as dehydroepiandrosterone (DHEA) are steroid precursors that do not show hormonal activity by themselves. Injectable anabolic steroids used in sports such as testosterone cypionate and nandrolone decanoate are esters of the parent steroid and are called pro-drugs since they are not active in the ester form. After administration and uptake the esters and pro-hormones are metabolized into biologically active androgens and estrogens as is illustrated in Figure 3 (Labrie et al. 1998; Lukacik et al. 2006). Therefore, feed and sport supplements containing such esters and pro-hormones have the potential to enhance the levels of natural occurring steroids and can thus be misused.

Chemical detection methods have the drawback of detecting only targeted compounds of interest. However, these supplements may contain anything ranging from the steroids themselves to esters, pro-hormones and possibly products resulting from metabolic conversions. Therefore, as an alternative screening for unknown steroids an in vitro reporter gene bioassay is used for bioactivity testing. Such bioassays expressing either the human androgen or progesterone receptor have been used to study the hormonal properties of synthesized tetrahydrogestrinone (THG), a designer drug (Death et al 2004). More recently, simple yeast-based reporter gene bioassays have been developed for trace analyses of estrogens as well as androgens (Bovee et al. 2004a, 2004b). Following metabolic activation using an S9 liver extract, inactive pro-hormones will also be detected with such a bioassay, as will inactive steroid esters following an enzymatic hydrolysis. Finally, considering that most feed supplements and herbal mixtures are of vegetable origin conjugated products as glycosides can be detected also. While some conjugated products still show some remaining androgenic activity, these conjugates can be converted back into bioactive steroids following a simple enzymatic deconjugation. Since the pro-hormones may theoretically also exist as their glycosides it is also required to perform multiple conversions, e.g. an enzymatic deconjugation followed by a metabolic activation, to include such products. An example of the (potential) testing scheme to include such compounds is given Table 1.

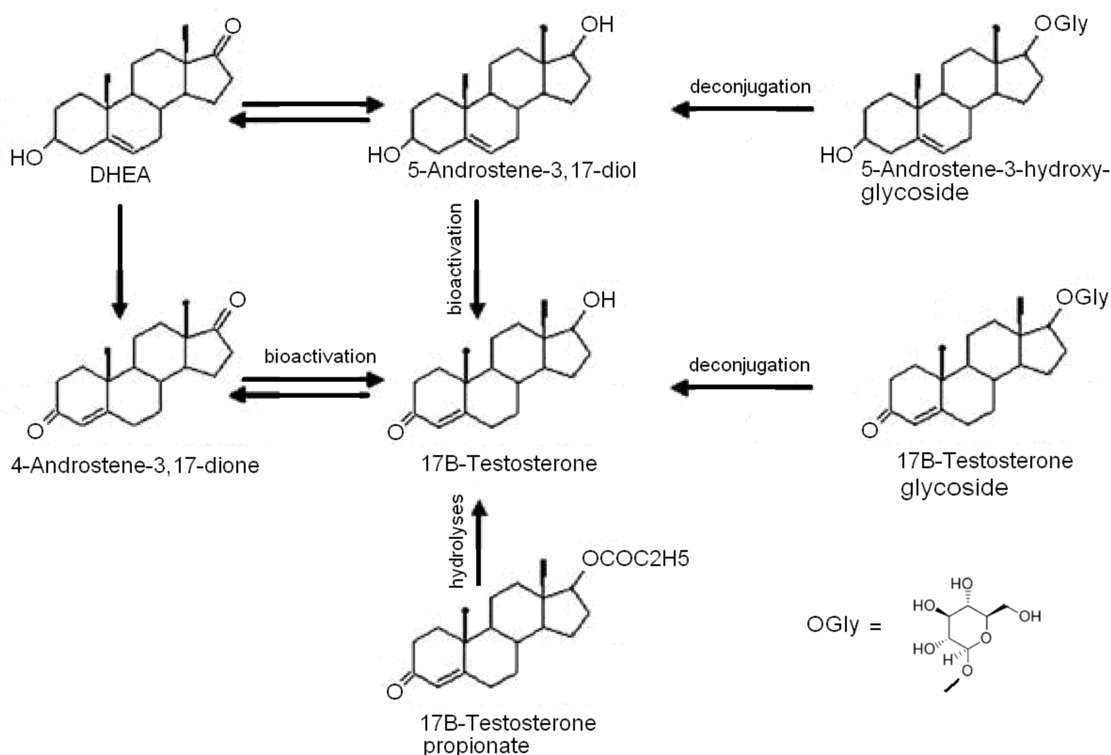



*Figure 3. Conversions of hormone esters (17B-testosterone propionate), hormone glycosides (17B-testosterone glycoside), pro-hormones (DHEA, 4-androstene-3,17-dione, 5-androstene-3,17-diol) and pro-hormone glycosides (5-androstene-3-hydroxy-17-glycoside) to the bioactive 17B-testosterone by enzymatic hydrolysis, enzymatic deconjugation, metabolic activation, and combinations thereof.*

*Table 1. Bioassay testing scheme for the detection of hormones, pro-hormones, their glycoside conjugates and esters. Only the first five are considered relevant.*

| Name | Sample treatment before bioassay |
|---|---|
| androgen | direct bioassay |
| glycoside androgen | deconjugation – bioassay |
| ester androgen | hydrolysis – bioassay |
| pro-androgen | S9 activation (S9) – bioassay |
| glycoside pro-androgen | deconjugation – S9 activation – bioassay |
| *ester pro-hormone* | *hydrolysis – S9 activation – bioassay* |
| *glycoside ester androgen* | *deconjugation – hydrolysis – bioassay* |
| *glycoside ester pro-androgen* | *deconjugation – hydrolysis - S9 activation – bioassay* |

The results of the androgen bioassay on the "cold-case" samples are summarized in Table 2. The direct androgen bioassay test did not result in a response of the cells, i.e. no direct hormonal activity was observed in vitro. Exceptions were the two digestamin samples that both reacted toxic towards the cells, even after 10-fold dilution of the sample extract. Digestamin is a herbal mixture containing

ginger root, fennel seed, rosemary, peppermint, marshmallow, vitamins and minerals. Herbal mixtures are sometimes added to food and feed as a anti-bacterial preservative to improve the shelf life of those products. A consequence of these anti-bacterial properties is that concentrated herbal extracts sometimes react toxic towards the cells in a bioassay. The test for pro-androgens, where the sample extract is incubated with an S9-mix prior to testing, was negative for all samples while the control samples were positive, indicating that no pro-androgens were detected in the samples.

For the detection of androgen esters an enzymatic hydrolysis was applied to the sample extracts prior to testing. While the control samples were positive, indicating a good performance of the test, the results for the samples were negative or inconclusive as indicated in Tables 2 and 3 by a $^{+}$/- sign. Such a result can be found if samples contain residues of active compounds, or if structurally different compounds are present that show a low biological activity. An example of this is a material to which an androgen ester is added. Since the androgen ester preparation often contains some free androgen residue as a result of incomplete esterification during synthesis, a direct androgen bioassay of the sample material may show a low response. An alternative may be that the sample material contains a pro-hormone or steroid derivatives like a steroid ether. Depending on the circumstances small fractions of these normally biologically inactive compounds may hydrolyze or metabolically activated resulting in a low response in the bioassay. The $^{+}$/- results for the androgen ester bioassay may for instance be the result of the presence of a pro-hormone ester or a glycoside ester androgen in the sample material. Although an inconclusive is an indication for the presence of an androgen-like compound, they are not representative for the compound group tested for, i.e. for that specific group they are considered negative and not further investigated. Androgen glycosides were determined following enzymatic deconjugation and as for the esters the results were negative for all "cold-case" samples. Finally, the test for pro-androgen glycosides, where two consecutive conversions are required prior to the androgen bioassay, showed only negative results. The conclusion of these tests is that none of the “cold-case” samples contain compounds with androgenic activity or compounds that produce androgens following metabolic activation.

A second group of samples that were tested were herbal mixtures and sport supplements. These samples were received from sport schools through intermediates. For the sport supplements the sample amounts were limited and therefore the test for pro-androgen glycosides was omitted. While the herbal mixtures consist of recognizable herbal material in capsules, the sport supplements are a white powdery material in colorful capsules. In this sample series the direct androgen bioassay showed a positive response with one of the samples, Herb A4. Logically, the bioassay results of this sample for the androgen esters and glycosides are therefore also positive. Pro-androgens were not determined in this sample. Another positive response was found for the sample Herb A2, in the test for androgen esters, e.g. after enzymatic hydrolysis of the sample extract. Apart from this sample the results of most other samples for androgen esters were negative or the sample extracts reacted toxic. For the androgen glycosides no positive results were found with the exception of sample Herb A4, which of course reacts positive because it was already positive in the direct androgen bioassay. From the results in Tables 2 and 3 it was decided to further investigate the samples Herb A2 and A4, and the sport supplements S1 to S4. Unfortunately, the available amount of the four sport supplements did not allow further bioassay testing so that research was limited to chemical analysis of the sample extracts. Of course, the bioassay results do not provide the chemical identity of the bioactive substance and this has to be elucidated using screening methods aimed at the identification of the unknown androgenic compounds in samples Herb A2 and A4.

*Table 2. Results of the androgen bioassay of suspected feed and feed supplements, the "cold cases". See the text for explanation of the signs.*

| RIKILT-code | Sample description | Androgens | Pro-androgens | Androgen esters | Androgen glycosides | Pro-androgen glycodides |
|---|---|---|---|---|---|---|
| 200141597 | feed | - | - | - | - | - |
| 200141598 | feed | - | - | $^{+}$/- | - | - |
| 200144635 | feed bigbag | - | - | $^{+}$/- | $^{+}$/- | - |
| 200144690 | supplement for hypophysis activity | - | - | $^{+}$/- | - | - |
| 200145516 | cod-liver oil | - | - | - | - | - |
| 200145518 | herbal mixture | - | - | - | - | - |
| 200146583 | herbal mixture | - | - | - | - | - |
| 200146585 | herbal mixture | - | - | - | - | - |
| 200146589 | herbal mixture | - | - | $^{+}$/- | - | - |
| 200148736 | all mash | - | - | $^{+}$/- | - | - |
| 200166957 | feed | - | - | $^{+}$/- | $^{+}$/- | - |
| 200207042 | herbal mixture | - | - | - | - | - |
| 200207177 | digestamin piglet | tox | - | $^{+}$/- | - | - |
| 200207178 | digestamin fattening | tox | - | $^{+}$/- | $^{+}$/- | - |

*Table 3. Results of the androgen bioassay of herbal and sports supplements. See the text for explanation of the signs.*

| Product name | Sample description | Androgens | Pro-androgens | Androgen esters | Androgen glycosides | Pro-androgen glycodides |
|---|---|---|---|---|---|---|
| Herb A1 | herbs in brown capsule | - | - | $^{+}$/- | - | nb |
| Herb A2 | herbs in brown capsule | - | - | + | - | nb |
| Herb A3 | herbs in orange capsule | - | - | $^{+}$/- | tox | nb |
| Herb A4 | herbs in orange capsule | + | nb | + | + | nb |
| Suppl. S1 | white powder in capsule | tox | - | $^{+}$/- | $^{+}$/- | nb |
| Suppl. S2 | white powder in capsule | $^{+}$/- | - | tox | $^{+}$/- | nb |
| Suppl. S3 | white powder in capsule | tox | tox | tox | tox | nb |
| Suppl. S4 | white powder in capsule | tox | nb | nb | - | nb |

## 3.2 Bioassay guided fractionation of positive samples and UPLC-TOFMS identification of the unknown, bioactive compounds

To pinpoint the bioactive unknown compounds in the positive samples of the previous paragraph, bioassay-guided fractionation as illustrated in Figure 1 in the introduction was used. The sample extracts of Herb A2 and Herb A4 were analyzed using UPLC/TOFMS and fractionated using an HPLC system equipped with dual fraction collection system with 96-well micro-titre plates. A part of the chromatogram of the extract of Herb A2 is shown in Figure 4. The results of the androgen activity in each collected well is shown directly below each chromatogram in the form of a biogram. The negative peaks in the chromatogram result from correction for the blank control samples. The biogram of Herb A2 in Figure 4 shows a small positive response in well numbers 54 and 63, correlating with retention times around 15.4 and 17.7 min. This is in agreement with the original testing for androgenic activity where sample Herb A2 gave a positive, but low androgenic response.
Although the active fractions of sample Herb A2 collected in wells 53, 54, 55 and 63 were also analyzed separately using UPLC/TOFMS, the original unfractionated chromatogram was used for identification of the active unknown compounds.  This approach was followed because the low response in sample A2 and the dilution of the extract as a consequence of the fractionation procedure may lead to compound concentrations that can no longer be detected or identified with UPLC/TOFMS.

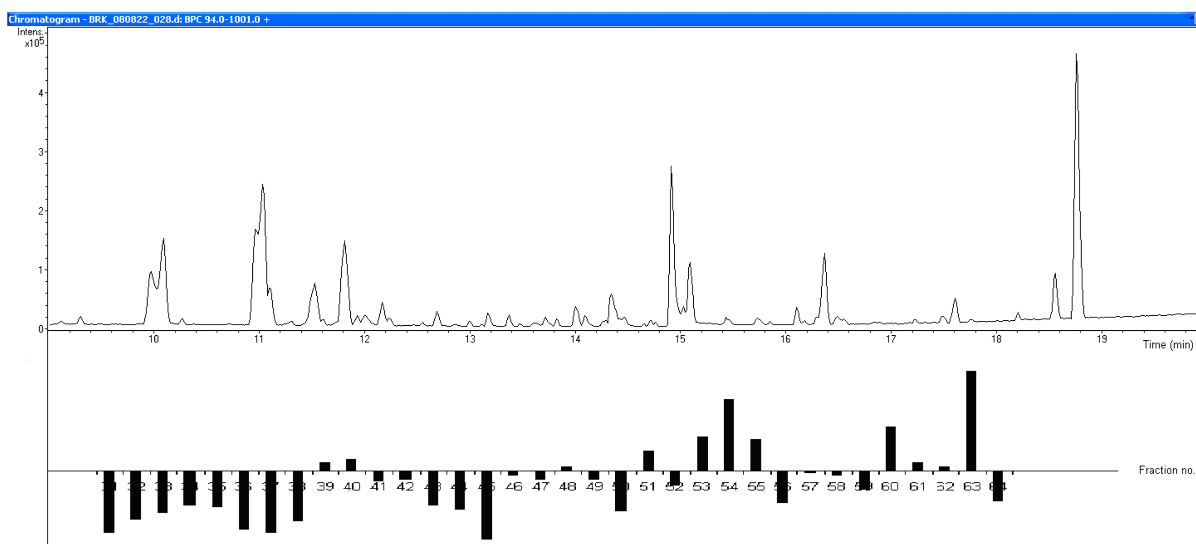



*Figure 4. Chromatogram (top) and biogram (bottom) of sample Herb A2. The biogram is corrected for the response of blank control samples, hence the negative peaks. The detected response in wells 54 and 63 is minimal.*

For the identification of unknown compounds in the chromatogram an accurate mass database containing approximately 40,000 compounds was used. The construction of this database will be discussed later. By visually inspecting all mass spectra around 15.5 min in the chromatogram of Herb A2, two ions were found that differ from the background, the first being m/z 407.2564 at 15.40 min, the second m/z 280.2677 at 15.45 min. The chromatogram and the mass spectrum at 15.40 min, without and with background subtraction, is shown in Figure 5 indicating the peak of the compound with m/z 407.2564.Visual inspection of the mass spectra around 17.7 min showed three ions differing

from the background, the first being m/z 399.2852 at 17.55 min, the second m/z 310.3150 at 17.60 min, and the third m/z 468.4448 at 17.76 min.

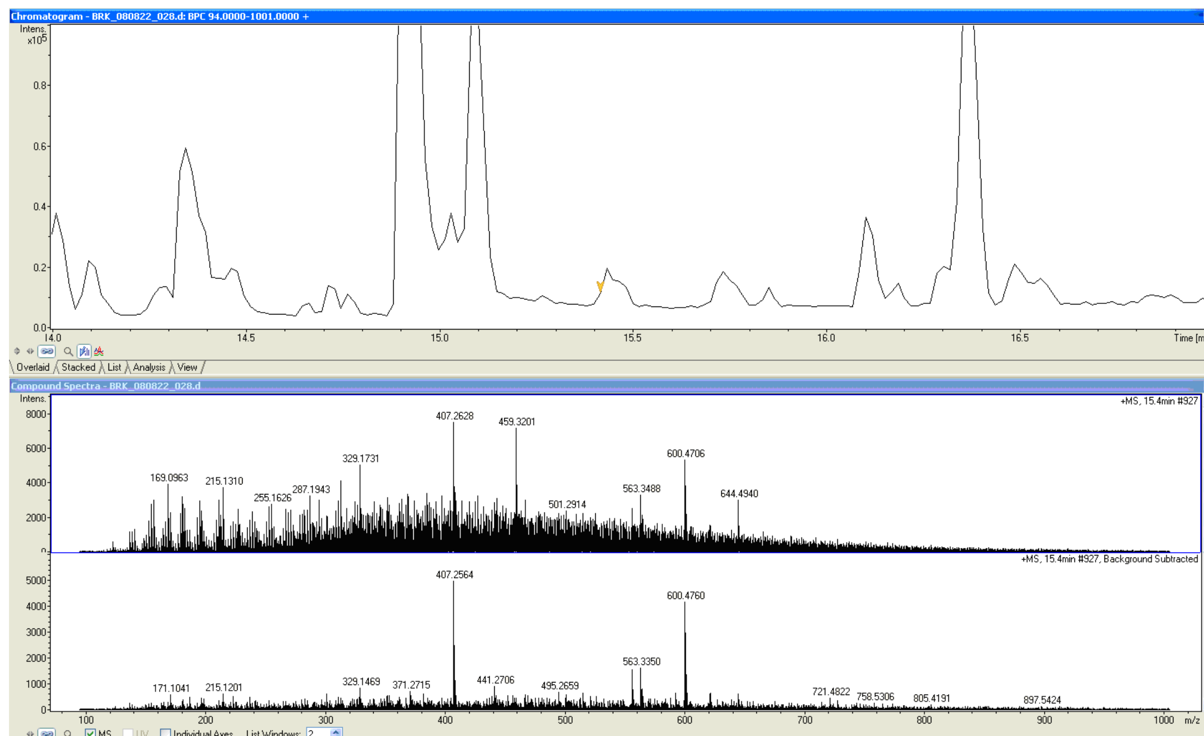



*Figure 5. Part of the chromatogram around the peak at 15.4 min suspected to contain un unknown compound with androgenic properties. The lower windows show the mass spectrum and background subtracted mass spectrum at 15.40 min showing ion m/z 407.2564 differing from the background.*

Using the database the compounds generating these ions were tentatively identified as:

| RT (min) | m/z (Da) | Tentative identification | Molecular formula | Δ m/z (mDa) | Δ m/z (ppm) |
|---|---|---|---|---|---|
| 15.40 | 407.2564 | Testosterone phenylacetate | $C_{27}H_{34}O_3$ | 1.7 | 4.2 |
| | | Durabolin (nandrolone phenylpropionate) | $C_{27}H_{34}O_3$ | 1.7 | 4.2 |
| | | Anticatabolin; Superanabolon | $C_{27}H_{34}O_3$ | 1.7 | 4.2 |
| 15.45 | 280.2677 | Linoleic acid amide | $C_{18}H_{33}NO$ | 4.2 | 15 |
| | | Farnesyl acetone (ammonium adduct) | $C_{18}H_{30}O$ | 4.2 | 15 |
| 17.55 | 399.2852 | Benzotest (Testodur) | $C_{26}H_{38}O_3$ | 4.1 | 10 |
| 17.60 | 310.3150 | no suggestion | | | |
| 17.75 | 468.4448 | no suggestion | | | |

It should be stressed that the identifications are no more than tentative identifications, e.g. preliminary identifications. The identity can only be confirmed by analyzing a standard of this compound using the same equipment and under the same conditions. If this standard shows the same retention time, mass spectrum and/or accurate mass and similar isotope ratios, the identity is "standard confirmed". It should also be pointed out that the database contains only protonated ions for the positive mode and that identifications therefore will always have the form $[M+H]^+$. Generally, electrospray ionisation (ESI) results in protonated molecules $[M+H]^+$ molecules in the positive mode and in deprotonated $[M-H]^-$ molecules in the negative mode. However, several adduct ions such as $[M+Na]^+$, $[M+K]^+$, and $[M+NH_4]^+$ can be formed in addition to $[M+H]^+$. Carboxyl or carbonyl ether and ester groups are believed to be responsible for binding the alkali metal ions originating from the matrix while ammonium ions result from addition of ammonium acetate or -formate to the LC mobile phase. The formation of such adducts depends on many factors as the presence of ions, the type of organic molecules, the type of MS instrument, the ionisation and the geometry of the ionisation chamber (Li et al. 2002; Mortier et al. 2004).

Ion m/z 280.2677 was tentatively identified as linoleic acid amide, an endogenous fatty acid primary amide found in plants together with other fatty acid amides like oleamide. Nowadays, these amides are produced on a large scale and are useful as fiber lubricants, detergents, flotation agents, textile softeners, antistatic agents, wax additives, and plasticizers but some of them have also specific biological functions. While simple amides of fatty acids were shown to possess an angiogenic factor (angiogenesis is the process involved in the growth and development of blood vessels as well as in wound healing), no mention of androgenic activity was found in the literature (Wakamatsu et al. 1990). However, taking into account the possible adduct formation due to the use of ammonium formate in the LC mobile phase, ion m/z 280.2677 could also be a $[C_{18}H_{30}O+NH_4]^+$ adduct. According to the database possible identifications for $C_{18}H_{30}O$ are 2,4,6-tri-t-butylphenol or farnesyl acetone. 2,4,6-Tri-t-butylphenol is a synthetic compound used in the production of antioxidants for rubbers and plastics and as an lubricating agent in the transport sector and therefore not likely to be found in herbal preparations. Farnesyl acetone is a terpenoic compound used in the manufacturing of fragrance and flavor concentrates of all types and produced in plants from acyclic carotenoids. Considering this natural origin farnesyl acetone could also be present in these samples. However, for an ammonium adduct loss of the neutral molecule $NH_3$ resulting in the protonated $[C_{18}H_{30}O+H]^+$ molecule is a major process. Inspection of the mass spectrum shows no clear sign's of neutral $NH_3$ loss, suggesting that the tentative identification as linoleic acid amide is most likely.

Ion m/z 407.2564 was identified as testosterone phenylacetate or Durabolin, Anticatabolin and Superanabolon. The latter three are all common names for nandrolone phenylpropionate. Nandrolone is a modification of testosterone (methyl-group removed from the 19th position) known as 19-nortestosterone. Nandrolone is sold commercially as its decanoate ester (Deca-Durabolin) and less commonly as a phenylpropionate ester (Durabolin). All identifications are esters, having the same molecular formula and can not be further distinguished unless the retention times under the conditions of the analyses are known. The finding of an androgen ester is supported by the primary test results that indicated that no direct acting androgen was present, but a positive response after enzymatic hydrolysis was shown indicating the presence of an androgen ester. As mentioned previously the identification are only tentative and should be confirmed by the analysis of a standard of the compound or the sample should be analyzed using a confirmatory method. In this case the identity was confirmed by the analysis of a sample of Herb A2 using RIKILT procedure RSV-A1025, a qualitative

confirmation method for steroid esters. The identity was standard confirmed as nortestosteron phenylpropionate and quantified as a concentration of approximately 0.2 mg/kg product. The latter information is of some interest because the bioassay test results of the primary test and of the 96-well plates from the fractionation showed that this concentration is close to the detection limit of the bioassay for androgen esters. For androgens themselves the detection limit may be lower because no enzymatic hydrolysis is needed. Although it is believed that the recovery from enzymatic hydrolysis and de-conjugation procedures are generally sufficient (e.g. >70%), incidentally low results can not be excluded. In addition, the androgenic potency and thus the sensitivity of the bioassay will be different for different compounds. Taking these factors into account detection limits of this method will most likely be in the range of 0.1 to 1 mg androgen/kg product.

Ion m/z 399.2852 was identified as Benzotest with Testodur and Virex-retard as alternative names. These are all trivial names for testosterone hexahydrobenzoate. It is known as a long-acting testosterone ester that was already tested in the '50s (Guiard 1956; Feyel-Cabanes et al. 1958) and has more recently been used in medical treatments and been administered to mares (Landier et al. 1984; Bonnaire et al. 1995). More recent information about the use of this testosterone ester was not found. The database did not produce any suggestions for ions m/z 310.3150 and 468.4448. The compounds in the database closest to these ion masses show a mass deviation Δ m/z of 50 mDa (100-150 ppm) and since these deviations are more than 10 times higher than those for other compounds they are considered unlikely. The database is limited in the sense that it only contains existing compounds and does not provide theoretical molecular formulas for accurate masses. However, if theoretical molecular formulas are calculated for the two ions using a mass deviation window of 10 mDa, four possible molecular formulas $C_{20}H_{40}NO$, $C_{18}H_{38}N_4$, $C_{19}H_{40}N_3$ and $C_{21}H_{42}O$ are found for ion m/z 310.3150 while more than 20 are found for ion m/z 468.4448. Of these, the molecular formulas $C_{30}H_{61}OP$, $C_{28}H_{59}N_3P$, $C_{31}H_{61}Cl$ and $C_{32}H_{56}N_2$ fall within a 1 mDa mass deviation window. Although further selections can be made using measured isotope ratios, and the number of rings and double bonds in the molecule can be calculated from the molecular formula, in this study we restricted the identifications to the accurate mass database.

Summarizing, the presence of nortestosteron phenylpropionate in sample A2 was in part responsible for the observed bioactivity in extracts of A2 following enzymatic hydrolysis. The peak of this hormone-ester was identified at 15.5 min in the A2 extract chromatogram and it's presence was standard confirmed by an independent LC/MSMS analysis containing an external standard of this compound. Bioassay guided fractionation indicated the presence of a second hormone-ester that was tentatively identified as testosterone hexahydrobenzoate, more commonly known as Testodur. Information about this compound is rare and dates from the 50's and 80's of the previous century, it's identity remains to be confirmed.

The chromatogram and biogram of sample Herb A4 is presented in Figure 6 and shows strong responses in well numbers 25 and 26, correlating to a retention time of 7.0 min in the sample chromatogram. Following fractionation of the sample extract of Herb A4 the fractions of well 25 and 26 showed a clear response in the bioassay and therefore these fractions were analyzed using UPLC/TOFMS. As a control, a blank extract (complete method but no sample) was also fractionated and the wells 25 and 26 of this blank were analyzed with UPLC/TOFMS in the same series. Figure 7 shows a part of the chromatogram of well 25 of the blank control (upper chromatogram) and of well 25 of the fractionated sample extract of Herb A4 (second chromatogram). Comparison shows that

there are two peaks in the sample extract that appear not to be present in the blank control. The first of these is found at retention time 7.05 min with m/z 303.2320 and the second at retention time 7.10 min with m/z 433.1491.

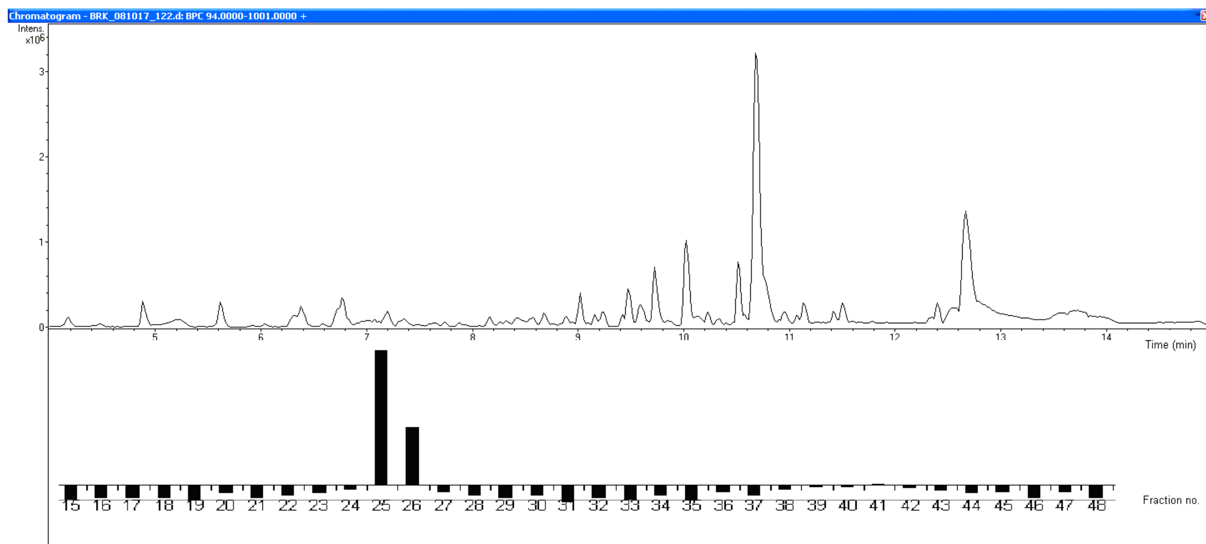



*Figure 6. Chromatogram (top) and biogram (bottom) of sample Herb A4. The biogram is corrected for the response of blank control samples, hence the negative peak's. The detected response in wells 25 and 26 show a clear positive results relative to the other wells.*

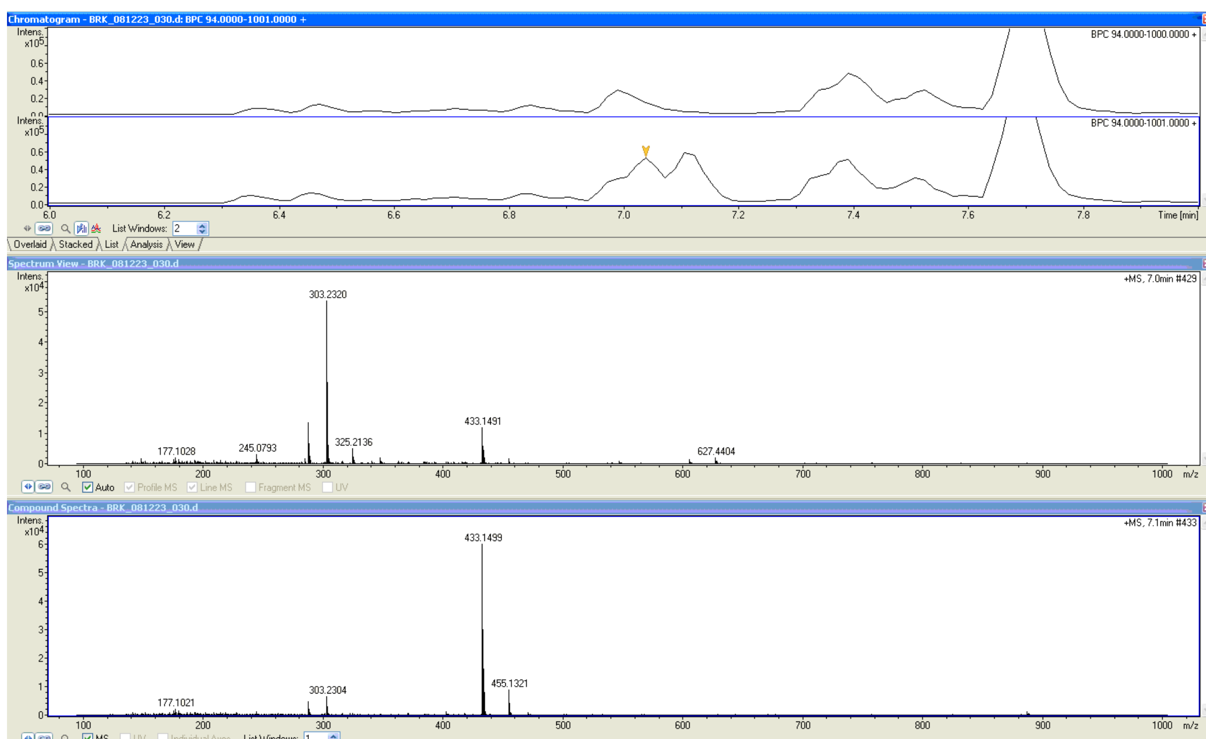



*Figure 7. Part of the chromatograms of well 25 after fractionation of a blank control and sample extract of Herb A4. Two peaks were identified in the sample extract that are not present in the blank control. These peaks are found at retention time 7.05 and 7.10 min and there respective mass spectra are shown below the chromatograms.*

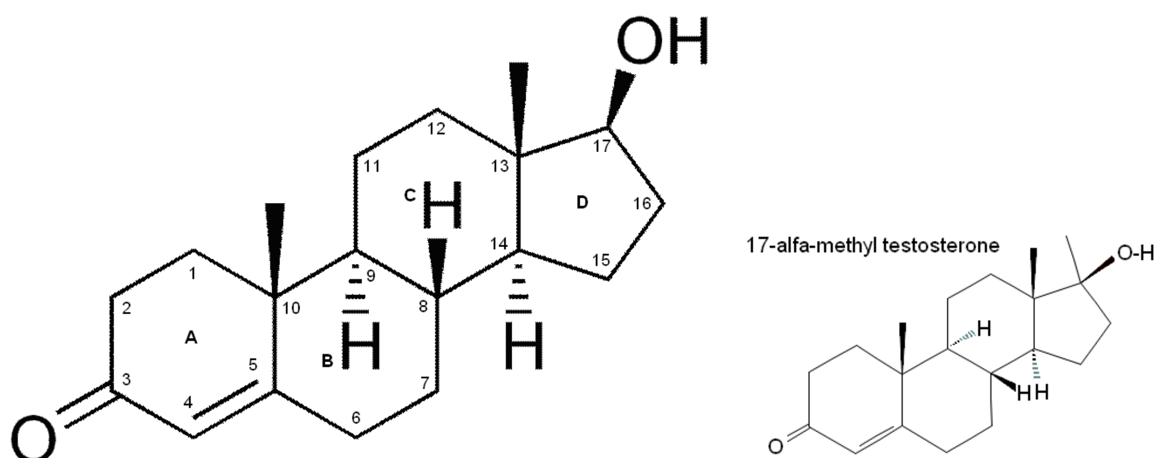
Using the accurate mass database these peaks were identified as:

| RT (min) | m/z (Da) | Tentative identification | Molecular formula | Δ m/z (mDa) | Δ m/z (ppm) |
|---|---|---|---|---|---|
| 7.05 | 303.2320 | NSC307483; Spiro(androstan-3,2'-oxiran)-17-one ... | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | Usic acid; d-Pimaric acid | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | Abietic acid; Abietate | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | MMHDA; 4a-Methylene-17-methyl-A-homo-B,19-dinorandrost-9-ene-3,17-diol | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | 3,4-Secotrachylobanoic acid | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | Beyer-15-en-18-oic acid | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | **3-HMAN; 3-Hydroxy-1-methyleneandrostan-17-one** | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| 7.05 | 303.2320 | Tetrahydronorethindrone; 19-Norpregn-20-yne-3,17-diol | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | Trachyloban-19-oic acid; Trachyloban-18-oic acid, (4beta)- | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | **Mibolerone** | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | **Stenbolone** | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | **4-Methyltestosterone** | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | **7-alpha-Methyltestosterone** | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | **17.alpha.-Methyltestosterone** | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | **Methenolone** | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | Teideadiol | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | **Oxendolone; Prostetin** | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |

| RT (min) | m/z (Da) | Tentative identification | Molecular formula | Δ m/z (mDa) | Δ m/z (ppm) |
|---|---|---|---|---|---|
| | | **RMI-12936** | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | Icosapent; Timnodonic acid | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | **17-O-Methyl testosterone ether** | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | **Nilevar; Solevar** | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | Peretinoin; Acyclic retinoid | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | 14(R)-Hydroxy-retro-vitamin A | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | Hepaxanthin; Vitamin A epoxide | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | 5-Hete lactone; 5-Hydroxyeicosatetraenoic acid lactone ... | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | 3-Hydroxyretinol | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | 5,6-dehydro AA; 5,6-dehydroarachidonic acid | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | 5,7,9,14,17-Icosapentaenoic acid; 5,7,9,14,17-Eicosapentaenoic acid | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | Asperketal B | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| | | 5-Etienic acid; 5-Androstene-17beta-carboxylic acid | $C_{20}H_{30}O_2$ | 0.15 | 0.5 |
| 7.10 | 433.1499 | PD 140248; 1,8-Naphthyridine-3-carboxylic acid, 7-(3-(1-aminoethyl-1-pyrrolidinyl))-1-(2,4-difluorophenyl)-6-fluoro-1,4-dihydro-4-oxo-, hydrochloride (10:11) | $C_{21}H_{19}F_3N_4O_3$ | 1.7 | 3.9 |
| | | Fpl 13210; Fpl-13210; 143305-97-9 | $C_{22}H_{29}BrN_2O_2$ | 1.7 | 3.9 |
| | | Podophyllic acid; Diaethyl-(2-chlor-1-(2,4-dichlorphenyl))-vinyl-phosphat | $C_{22}H_{24}O_9$ | 0.6 | 1.4 |
| | | 3-Hptmf; Hepta-3; Ambap2359 ... | $C_{22}H_{24}O_9$ | 0.6 | 1.4 |

| RT (min) | m/z (Da) | Tentative identification | Molecular formula | Δ m/z (mDa) | Δ m/z (ppm) |
|---|---|---|---|---|---|
| | | Rubropilosin; 2-Hydroxy-4-hydroxymethyl phenyl 6-O-cinnamoyl-beta-D-allopyranoside | $C_{22}H_{24}O_9$ | 0.6 | 1.4 |

For m/z 303.2320 the database finds 30 possible identities, all having the molecular formula $C_{20}H_{30}O_2$. Information about these compounds can be found in the PubChem database on the internet (PubChem 2009), or from different sources on the internet using a search engine like Google. Since we know that the unknown we are looking for is an androgen we can exclude most of the identities in the list with the exceptions of the bold printed compound names. Of these Mibolerone, Stenbolone, 4-, 7- and 17-methyltestosterone, Methenolone and 17-O-methyl testosterone ether, Nilevar, 3-HMAN and RMI-12936 are known or likely androgens. The first six of these compounds are all methyltestosterones as shown in the molecular structure of testosterone in Figure 8. In 17-O-methyl testosterone ether, or 17-methoxy testosterone, the hydrogen of the hydroxyl group at the 17-position is replaced by a methyl-group. Since the human body has no enzymes to remove the ether bond, 17-O-methyltestosterone ether is about the only testosterone ether showing anabolic activity (Solo et al. 1975). In Nilevar the methyl-group at the 10-position is replaced by a hydrogen while an ethyl-group is bound at the 17-position together with the hydroxyl-group (Koert et al. 2008). Oxendolone is mentioned in the literature as an anti-androgen (Okada et al. 1988). RMI-12936, also known as 7-methyl-17β-hydroxy-androst-5-en-one was tested for androgenic activity in mouse kidney and was bound by the renal androgen receptor with a relative affinity approximately one-third that of testosterone (Bullock et al. 1978). 3-HMAN (3-hydroxy-1-methyleneandrostan-17-one) was found to be a major urinary metabolite of methenolone acetate, an androgen ester, but nothing is mentioned about any androgenic acivity (Curehunter 2009).



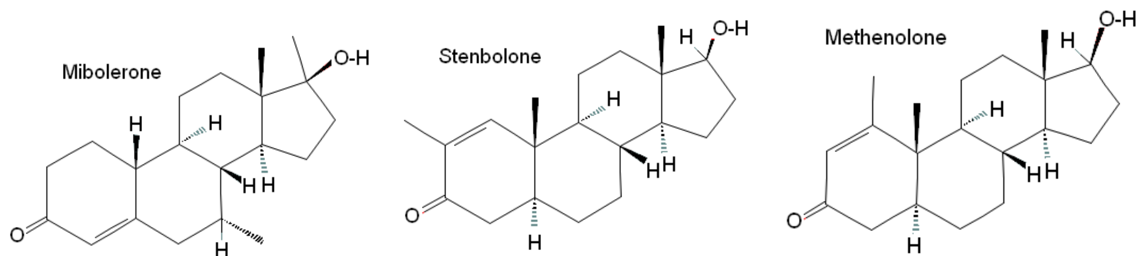



*Figure 8. Testosterone structure and numbering, and the structure of four methyl testosterone isomers. Compared to testosterone, 4-, 7- and 17-methyl testosterone have an additional methyl group in their respective positions. Mibolerone has an additional methyl-group at the 7- and 17-position and a proton instead of a methyl-group at the 10 position. Stenbolone has an additional methyl-group at the 2-position and the 4,5-double bond has moved to the 1,2-position. Methenolone has an extra methyl-group is at the 1-position and the 4,5-double-bound has moved to the 1,2-position.*

For m/z 433.1499 the database finds 5 possible identities. PD 140248 is a synthetically produced broad-spectrum antimicrobial agent that has been demonstrated to have excellent in vitro activity against gram-positive organisms such as staphylococcus and streptococcus infections (Steiert et al. 2000). The identification as Fpl-13210 (CAS 143305-97-9) with molecular formula $C_{22}H_{29}BrN_2O_2$ can not be correct since the $[M+H]^+$+2 isotope peak with equal intensity as the $[M+H]^+$ peak is missing in the mass spectrum. Podophylic acid, or podophyllin is a resinous powder obtained by precipitating an alcoholic tincture of the rhizome of American Mayapple (Podophyllum peltatum) by means of water acidified with hydrochloric acid. These compounds belong to the podophyllotoxins, a group of compounds with potential anti-tumor activity (Tang et al. 2009). 3-Hptmf (CAS 1178-24-1) stands for 3,3',4',5,6,7,8-Heptamethoxyflavone and no further information was found about this compound. Finally, about ruboprilosin no other information was found than that it is related with the production of carbohydrates in nature (Perold et al. 1973).

Evaluating the possible identities of both peaks in the chromatogram in Figure 6, it is concluded that the peak at 7.05 min in the chromatogram with m/z 303.2320, is most likely responsible for the observed androgenic response. This compound with molecular formula $C_{20}H_{30}O_2$, is probably a methyltestosterone though it is unknown which exact isomer. The identity was confirmed by the analysis of a sample of Herb A4 using a modified version of RIKILT procedure RSV-A1050, an analysis method for steroids including 17α-methyltestosterone using derivatization of the hydroxy group followed by GC/MSMS analysis. Different from the normal procedure the analysis was performed in the scan mode allowing the acquisition of full scan mass spectra which, together with retention time information, allow the confirmation of the identity of the compound. The identity was standard confirmed as 17α-methyltestosterone quantified at a concentration of approximately 4 mg/kg product. The results of the analysis of these herbal mixtures show that the procedure of bioassay guided fractionation in combination with UPLC/TOFMS analysis of sample extracts and extract fractions, is capable of pin-pointing and identifying the androgenic compounds. It should be added that the availability of an adequate accurate mass database is probably a pre-requisite for successful compound identification and this database will be the subject for the next paragraph.

## 3.3 Identifying compounds without primary reference standards

Apart from the herbal mixtures four sport supplements S1 to S4 were investigated with the bioassay. However, the results of the bioassay tests were toxic or inconclusive for most extracts. Since the amount of test material was limited it was not possible to investigate these samples further with the bioassays. As an alternative approach it was decided to analyze the sample extracts with UPLC/TOFMS and use a large database to identify individual compounds in the products as far as possible. This accurate mass database was constructed based on compounds extracted from the PubChem database that can be found on the internet (PubChem 2009). Interestingly, the database not only contains the parent compounds but also many metabolites and conjugated compounds as steroid glucuronides, pro-hormones as DHEA and steroid esters as testosterone phenylacetate. For instance, using a filter and selecting all compound names containing the term “testosterone” results in 203 entries, a part of which is shown in the database "report form" shown in Figure 9. The database contains 167 entries including the term “glucuronides”, 42 entries containing the term “sulphate” but only 2 entries containing the term “glycoside”. It should also be noted that scientific compound names are not always used. As an example, compound names as Durabolin, Anticatabolin, Superanabolon and nandrolone phenylpropionate, all different names for the same compound, are listed separately in the database. However, the commonly used scientific name for this compound, nortestosteron phenylpropionate, is not. Another point is that with 40,000 entries in the range of 100 to 750 Da, there are many compounds with the same or almost the same mass and as a result more compounds will fall into a certain mass tolerance window than when a smaller database would be used. Isotopic pattern matching is a powerful criterion in addition to exact mass for compound identification and may be able to differentiate between compounds of (nearly) identical mass (Ojanperä et al. 2005), however, if the molecular formulas are identical this approach will not help. In the database the average number of hits with identical molecular formula is less than 2 with a median value of 1, indicating that in more than half of the occasions an accurate mass will produce a molecular formula with only one structural isomer. This is visualized by Figure 10 that presents the distribution of the number of isomers in the database showing that for 71% of the entries in the database there is only 1 isomer while for 90% of the data there are 3 or less isomers. Only for 1% of the entries (still 400 molecular formulas!) the number of isomers is in the range of 11 to 32.

| Exact Mass (M+H) Bruker TOF | Trivial Name | IUPAC Name | Molecular Formula | XlogP | PubChem Code |
|---|---|---|---|---|---|
| 275.200550 | nandrolone; 19-Nortestosterone; Nortesto | (8R,9S,10R,13S | C18H26O2 | 3.2 | CID:9904 |
| 276.195799 | 11-Aza-19-ntt; D-11-Aza-19-nortestostero | | C17H25NO2 | 0.8 | CID: 193813 |
| 277.216200 | 5-Dihydronandrolone; 5-Dihydro-19-norte | (5S,8R,9R,10S, | C18H28O2 | 3.9 | CID: 94202 |
| 285.184900 | 6,8(14)-Bisdehydrotestosterone; 17-Hydr | (9R,10R,13S)-1 | C19H24O2 | 2.0 | CID: 152665 |
| 287.200550 | Boldenone; Dehydrotestosterone; 1-Dehy | (8R,9S,10R,13S | C19H26O2 | 4.1 | CID: 13308 |
| 287.200550 | 6-Dehydrotestosterone; 6,7-Dehydrotesto | (8R,9S,10R,13S | C19H26O2 | 3.4 | CID: 17209 |
| 287.200550 | 6-Dehydrotestosterone; 6,7-Dehydrotesto | (8R,9S,10R,13S | C19H26O2 | 3.4 | CID: 17209 |
| 289.216200 | Epitestosterone; Isotestosterone; cis-Test | (8R,9S,10R,13S | C19H28O2 | 3.6 | CID: 10204 |
| 289.216200 | Testosterone-d3; Testosterone-d3 solutio | (10R)-17-hydrox | C19H28O2 | 3.6 | CID: 16220011 |
| 289.216200 | 1-testosterone; Delta1-dihydrotestosteron | (5S,8R,9S,10R, | C19H28O2 | 4.1 | CID: 236666 |
| 289.216200 | Retrosterone; Lumitestosterone; Retrotes | (8R,9R,10S,13S | C19H28O2 | 3.6 | CID:3034651 |
| 289.216200 | testosterone; Testostosterone; Homoster | 17-hydroxy-10,1 | C19H28O2 | 3.6 | CID:5408 |
| 289.216200 | Epitestosterone; Isotestosterone; cis-Test | (8R,9S,10R,13S | C19H28O2 | 3.6 | CID: 10204 |
| 291.195465 | 11-Oxatestosterone; 114683-04-4 | | C18H26O3 | 1.2 | CID: 195107 |
| 291.231850 | Androstanolone; dihydrotestosterone; An | (5S,8R,9S,10S, | C19H30O2 | 4.5 | CID: 10635 |
| 291.231850 | dihydrotestosterone; 17-Hydroxyandrosta | (5R,8S,9R,10R, | C19H30O2 | 4.5 | CID: 968803 |
| 293.191128 | 4-F-Ntt; 4-Fluoro-19-nortestosterone; Estr | (8R,9S,10R,13S | C18H25FO2 | 2.8 | CID: 150947 |
| 293.247500 | 4,5-Secodihydrotestosterone; 17-Hydroxy | 4-[(3S,3aR,5aR, | C19H32O2 | 5.2 | CID: 131285 |
| 295.206778 | 11beta-F-19-Nor-dht; 11-Fluoro-19-nordih | (8S,9R,10S,11S | C18H27FO2 | 3.2 | CID: 130697 |
| 301.216200 | NSC3356; 9(11)-Dehydro-17-methyl-testo | (8S,10S,13S,14 | C20H28O2 | 3.6 | CID: 220507 |
| 301.216200 | NSC18215; Testosterone, 6-dehydro-17-r | (8R,9S,10R,13S | C20H28O2 | 3.9 | CID: 227054 |
| 303.195465 | 11-Oxotestosterone; 11-Ketotestosterone | (8S,9S,10R,13S | C19H26O3 | 1.3 | CID: 104796 |
| 303.195465 | 11-Ketotestosterone; 11-Keto-testosteron | (8S,9S,10R,13S | C19H26O3 | 1.3 | CID:5282365 |
| 303.231850 | 4-Methyltestosterone; 4-METHYL TESTO | (8R,9S,10R,13S | C20H30O2 | 3.7 | CID: 236536 |
| 303.231850 | Isomer 201; 7-alpha-Methyltestosterone; | (7R)-17-hydroxy | C20H30O2 | 4.1 | CID: 24280 |
| 303.231850 | 7alpha-Methyltestosterone; 7-alpha-Meth | (7R,8R,9S,10R, | C20H30O2 | 4.1 | CID: 247942 |
| 303.231850 | Testosterone, 17-methyl-; 17.alpha.-Meth | 17-hydroxy-10,1 | C20H30O2 | 4 | CID:4160 |
| 303.231850 | 17-O-Methyl testosterone ether; 17-Metho | 17-methoxy-10, | C20H30O2 | 4.1 | CID: 538446 |
| 303.231850 | methyltestosterone; Mesterone; Testred .. | (8R,9S,10R,13S | C20H30O2 | 4 | CID:6010 |
| 304.227099 | Testosterone-3-oxime; Androst-4-en-3-on | (3Z,8R,9S,10R, | C19H29NO2 | 3.8 | CID:9577125 |

*Figure 9. Screenshot of a part of the database in Excel when a filter for the term “testosterone” was applied. Total number of entries found for the term "testosterone" is 203. Exact masses are typically calculated for the Bruker MicroTOF but are also available in the database for the Waters LCT.*

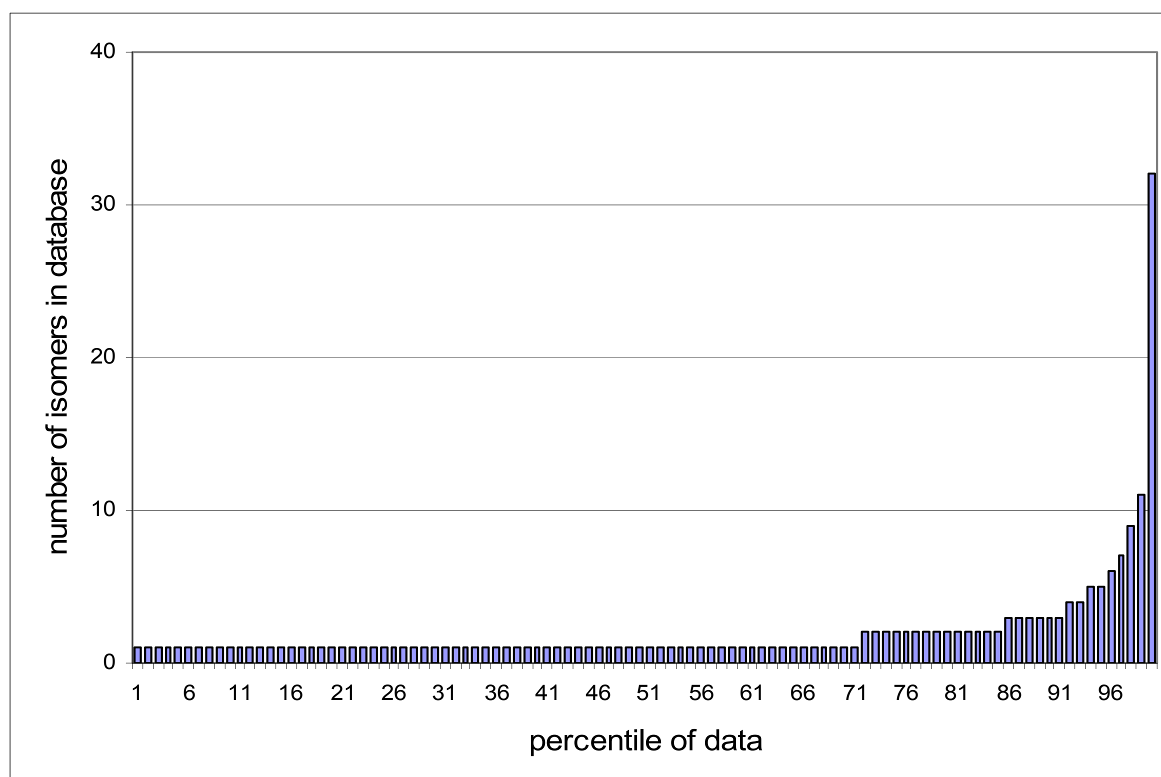



*Figure 10. Distribution of the number of isomers in the database showing a range of 1 to 32 isomers with a median of 1 isomer and >5 isomers for about 5% of the entries.*

The extracts of the sport supplements S1 to S3 (S4 was no longer available) were analyzed using UPLC/TOFMS and the accurate mass database was used to identify individual compounds in the products as far as possible. At first each chromatogram was searched manually. The search criteria for unknown (or untargeted) compounds were ±2 mDa for exact mass deviations, ±20% for the $[M+H]^+ +1$ or the $[M+H]^+ +2$ isotope peak. No retention time information was available. Finally, common sense and knowledge about the nature of the sample, in this case the search for androgens in sport supplements, was used to decide about the possible identity of a peak in the chromatogram. Figure 11 shows the chromatogram of sport supplement S1.

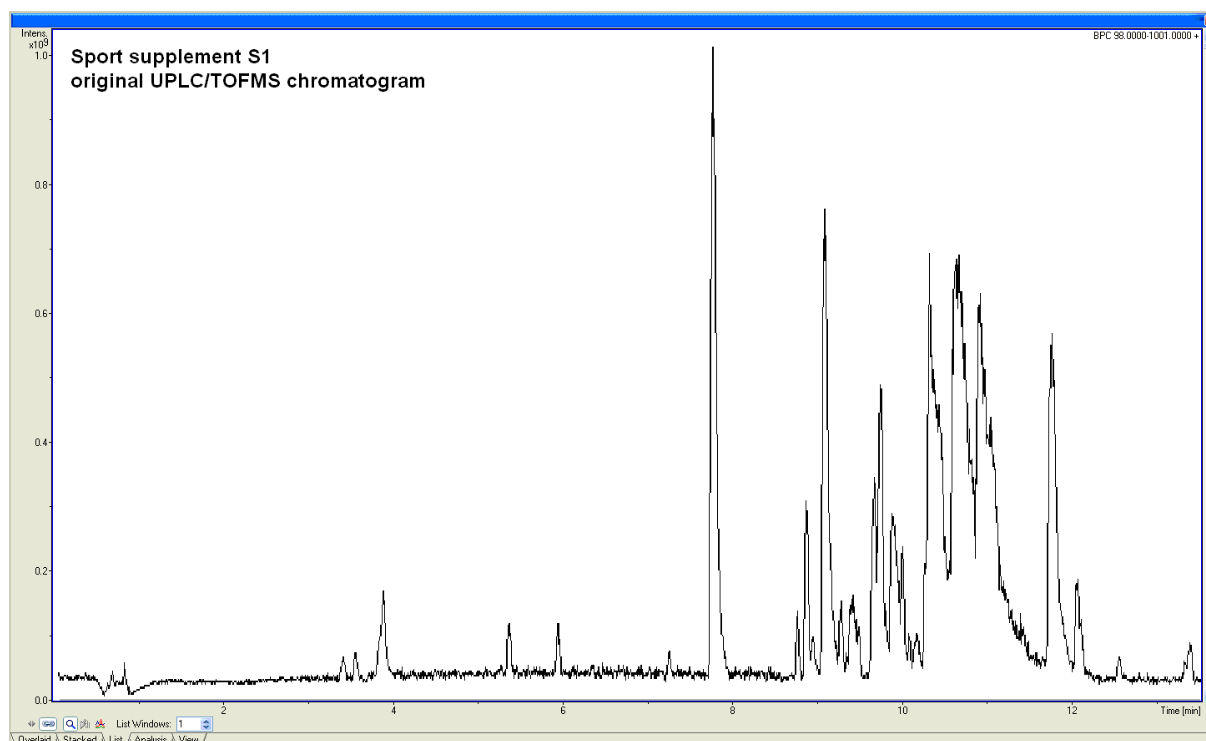



*Figure 11. Chromatogram of an extract of sport supplement S1.*

Although the results for the Herbal samples A2 and A4 in the previous section were obtained by manual investigation of the chromatograms, manual investigation without bioassay guided fractionation proved extremely laborious. While the use of UPLC/TOFMS generates an enormous amount of data allowing the identification of unknown compounds, depending on the matrix, this enormous amount of data also causes problems in identification of compounds. Manual searching of TOFMS data for unknown compounds of interest is laborious, firstly because peak picking in a typical TIC chromatogram of a UPLC/TOFMS analysis is difficult (see Figure 11), secondly because matrix interferences and overlapping peaks may seriously hamper the ability to obtain “clean” mass spectra, and thirdly because (large) component databases or libraries in UPLC/TOFMS analysis are virtually non-existent. For an efficient identification of unknowns elimination of matrix interferences and background noise is necessary requiring data reduction routines to “clean-up” the original chromatogram. A number of tools for pre-processing of MS data have been proposed in the literature (Fiehn et al. 2005; Broeckling et al. 2006; Luedemann et al. 2008) and some are commercially available from MS manufacturers such as Metabolynx™ (Waters) that was used previously in this study, and SieveTM (ThermoFischer Scientific). MetAlign™, an interface driven tool for hyphenated full-scan mass spectrometry data processing, has been developed in house at RIKILT (Lommen 2009).

While the main purpose of this software is the automated processing of MS-based metabolomics data, it is also capable of automatic format conversions, accurate mass calculations, baseline corrections, chromatogram alignment, peak-picking, and saturation and mass-peak artifact filtering of data sets, resulting in a 100 to 1000 fold data reduction. MetAlign$^{TM}$ is a freeware program that can be downloaded from the internet.

In this study MetAlign has been used to reduce the original UPLC/TOFMS data files by baseline correction, smoothing and elimination of detector and chemical noise. Figure 12 shows the same chromatogram as in Figure 11 but after data pre-processing with MetAlign. Since MetAlign identifies unique ion masses and removes noise the number of peaks in the chromatogram seems to increase, however, in reality these peaks were already there but obscured by chemical noise. The symmetrical peak shapes after pre-processing are inherent to the MetAlign software processing routines. In addition, the accurate mass database was placed in an Excel file in a special format that allows it to be used by Brukers Target Analyses$^{TM}$ software to search processed chromatograms for any of these compounds. It should be noted that this is a reverse-search process where each ion of interest in the database is extracted from the sample file and compared to the accurate mass in the database. This is a time-consuming process, but this is how one typically analyzes the data manually. The search criteria used to analyse the data files are the accurate mass, the SigmaFit$^{TM}$ and a minimum peak area. The SigmaFit is an algorithm developed by Bruker that indicates the exact numerical match between the theoretical (calculated from the elemental composition) and measured isotopic patterns. In this way the isotopic pattern (or ratio) can be used as an additional identification criterion (Ojanperä et al. 2005). Since the pre-processed chromatogram in Figure 12 contains a large number of peaks, each with his own accurate mass, this will potentially result in the same large number of molecular formulas. And since each molecular formula may result in multiple structural isomers, i.e. different compounds, this process may result in a number of identifications in the range of 100-1000. In this case the minimum area setting is used to limit the number of “identified” compounds at first. If required the method can be made more “sensitive” by lowering the minimum area allowing identifications of smaller peaks in the chromatogram. The pre-processed chromatograms of sport supplements S1, S2 and S3 have been processed in this way and the identifications reported here are limited to the compounds of interest in these samples, e.g. androgens or compounds that were suspected to improve performance in sports. In addition, since the database only contains the $[M+H]^+$ ions and no adducts like $[M+Na]^+$, $[M+K]^+$ and $[M+NH_4]^+$, the latter are also excluded from the search. In the pre-processed chromatogram of sport supplement S1 in Figure 12 a number of interesting peaks are marked with capital letters. These correspond to the identifications briefly discussed below. Keep in mind that these are no more than tentative identifications and confirmation by analysis of the actual standards under the same conditions is required to obtain certainty. Compounds of interest identified in the processed chromatograms of sport supplements S2 and S3 are found following Figures 13 and 14 respectively.

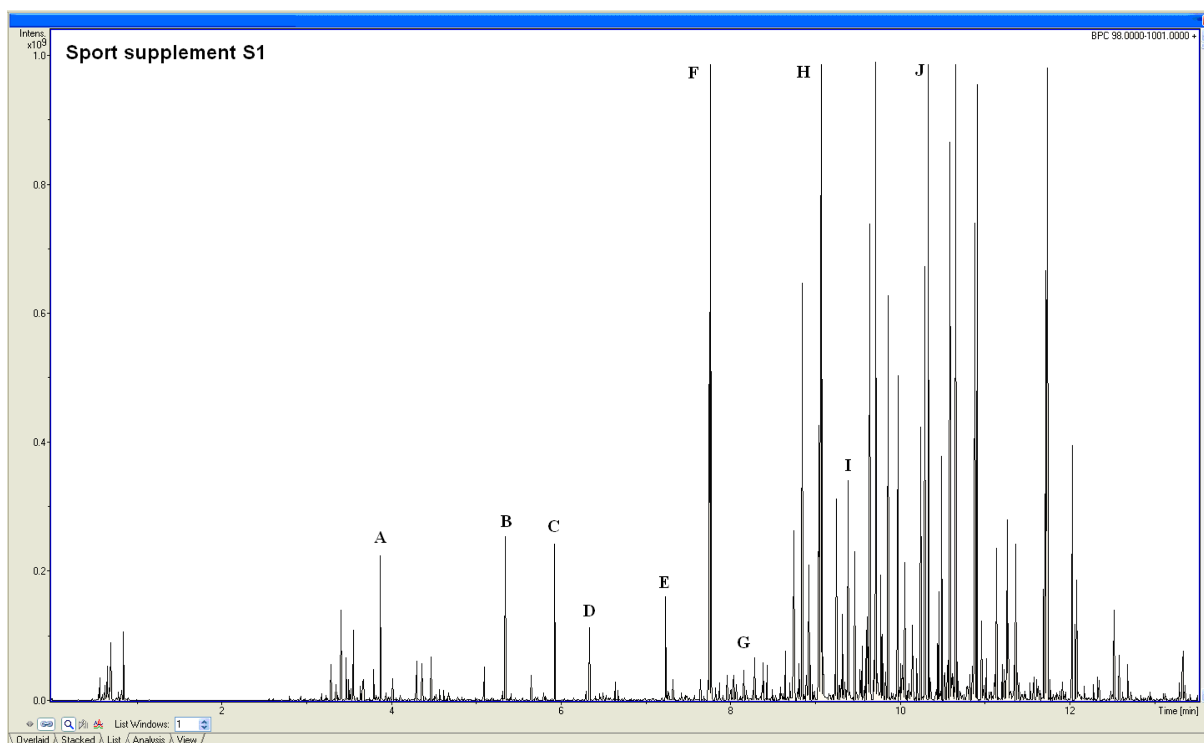



*Figure 12. Chromatogram of an extract of sport supplement S1 after pre-processing with MetAlign.*

A: RT = 3.81 min, m/z 329.0865, $C_{14}H_{16}O_9$. Identified as bergenin an isocumeric compound found in the Bergenia crassifolia (Siberian Tea), Astilbe thunberg (Ostrich Plume) and Ardisia japonica (Marlberry Bush) plants (Ojanperä et al. 2005). Bergenin aids in fat loss and healthy weight maintenance by opposing the lipogenic action of insulin and enhancing the lipolytic effects of norepinephrine. By preventing fat storage and stimulating fat burning, bergenin is used as a dietary weight-loss supplement.

B: RT = 5.35 min, m/z 289.2160, $C_{19}H_{28}O_2$. While the accurate mass matches with exactly one molecular formula, 9 possible identities or names were found. These include testosterone, but also epitestosterone, retrosterone, normethandrone, trestolone, dehydroepiandrosterone, benorterone, 3-hydroxy-4-androsten-17-one, androstane-3, androstanedione and 19-hado.

C: RT = 5.93 min, m/z 289.2159, $C_{19}H_{28}O_2$. Probably an isomer of peak B. Although the retention time and retention time difference between peaks B and C suggest a combination of testosterone and epi-testosterone, this is not likely since epitestosterone is the inactive epimer of testosterone and thus not likely to be present in an sport supplement.

D: RT = 6.34 min, m/z 188.1646, $C_{10}H_{21}NO_2$. Decanohydroxamic acid.

E: RT = 7.24 min, m/z 272.1279, $C_{16}H_{17}NO_3$. Identified as carbazomycin C or norcoclaurine with the latter being a biosynthetic precursor of thebaine and morphine (Loeffler et al. 1987).

F: RT = 7.77 min, m/z 286.1436, $C_{17}H_{19}NO_3$. Possible identifications are carbazomycin D, coclaurine, morphine or norcodeine.

G: RT = 8.04 min, m/z 301.2159, $C_{20}H_{28}O_2$. For this molecular formula the database reports no less than 21 possible isomers including as most notably dianabol, norgesterone and 17-ethylestradiol. Dianabol (methylboldenone) is an anabolic steroid released in the US in the early 1960's. It was used as an aid to muscle growth by bodybuilders until it was banned by the FDA. 17-ethylestradiol has a low anti-estrogenic activity.

H: RT = 9.06 min, m/z 251.2005, $C_{16}H_{26}O_2$. Identified as methyl farnesoate or octoxinol. Octoxinol, also known as Triton X-100, is a detergent sometimes used as a antihydroticum, a substance to reduce transpiration. However, since octoxinol are usually mixture with multiple ethoxy groups, its presence here as a single peak in the chromatogram is unlikely. Methyl farnesoate on the other hand is a natural terpenoid compound similar to juvenile hormones in insects and is itself a hormone in crustaceans (Laufer et al. 2005).

I: RT = 9.38 min, m/z 327.1960, $C_{21}H_{26}O_3$. Possibly moxestrol, 2-methoxyethinyl estradiol or 11-hydroxycannabinol, a metabolite of cannabinol more potent than the parent compound itself.

J: RT = 10.33 min, m/z 324.2895, $C_{20}H_{37}NO_2$. Peak identified as linoleamide MEA, a common compound in household and personal care products.

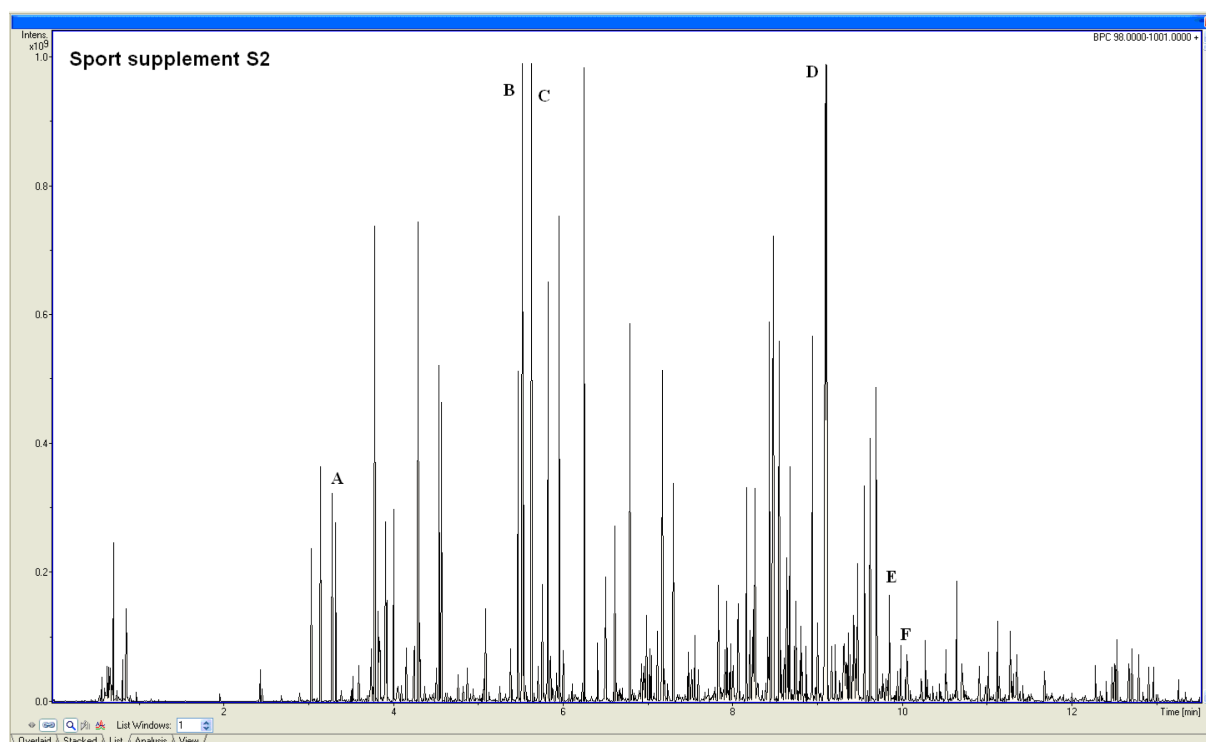



*Figure 13. Chromatogram of an extract of sport supplement S2 after pre-processing with MetAlign.*

A: RT = 3.28 min, m/z 181.0722, $C_7H_8N_4O_2$. This peak was identified as theobromine, also known as xantheose, a bitter alkaloid of the cacao plant found in chocolate.

B: RT = 5.52 min, m/z=352.1562, $C_{21}H_{21}NO_4$. Four compounds were reported by the database including zindoxifene, an anti-estrogen originally developed as a drug for the treatment of hormone-dependent mammary carcinomas (Schneider et al. 1991).

C: RT = 5.63 min, m/z 703.3059, $C_{32}H_{46}N_8O_6S_2$. This peak was identified as bisibutiamine, also known as sulbutiamine with brand name Arcalion. It is a synthetic derivative of thiamine (vitamin B1) and is available for over-the-counter sale as a nutritional supplement. Since this is a lipophilic compound a longer retention time is expected and the identification is unlikely.

D: RT = 9.11 min, m/z 417.2269, $C_{24}H_{32}O_6$. Possible identity is deoxyschisandrin, the composite of Schisandra chinensis, a famous Chinese medicine widely used as an anti-stress, anti-aging and neurological performance-improving herb (Fu et al. 2008).

E: RT = 9.75 min, m/z 359.2578, $C_{23}H_{34}O_3$. Compounds identified as the androgen esters 7-alpha-methyltestosterone propionate or testosterone isobutyrate. The longer retention time supports the idea that these are the more apolar esters in stead of the free steroids.

F: RT = 10.07 min, m/z 455,352, $C_{30}H_{46}O_3$. Although the database produced 8 isomers for this molecular formula, the most interesting was testosterone buciclate, a relatively new testosterone ester which has been synthesized and developed through the initiative of the World Health Organization (WHO) and the National Institutes of Health (NHI). It was purposely developed in the 1990s as a male contraceptive drug. Testosterone buciclate's androgenic potentials have been proven during its clinical testing as it showed more favourable pharmacokinetic characteristics than existing compounds, primarily because it is a slow release compound.

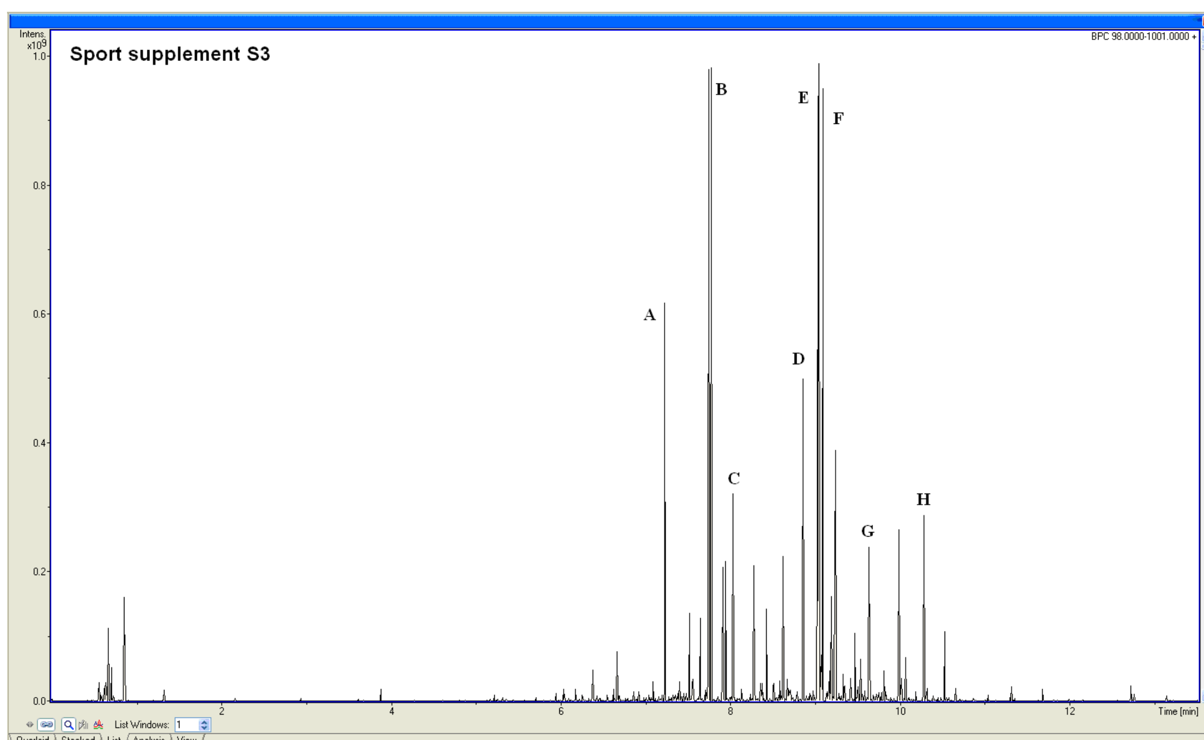



*Figure 14. Chromatogram of an extract of sport supplement S3 after pre-processing with MetAlign.*

A: RT = 7.23 min m/z 272.1284, $C_{16}H_{17}NO_3$. This peak was identified as norcoclaurine, carbazomycin C or normorphine (or demethylmorphine). See remarks peak E in Figure 12.

B: RT = 7.77, m/z 286.1433, $C_{17}H_{19}NO_3$. Coclaurine, carbazomycin D, morphine, norcodeine.

C: RT = 8.03 min, m/z 301.2155, $C_{20}H_{28}O_2$. Peak identified as methylboldenol (Dianabol). See remarks for peak G in Figure 12.

D: RT = 8.86 min, m/z 268.2271, $C_{16}H_{29}NO_2$. Identified as 1-lauroyl-2-pyrrolidinone or epilachnene, an alkaloid and natural macrolide.

E: RT = 9.03, m/z 251.2007, $C_{16}H_{26}O_2$. Methyl farnesoate, see remarks for peak H in Figure 12.

F: RT = 9.09, m/z 268.2272, $C_{16}H_{29}NO_2$. Identified as 1-lauroyl-2-pyrrolidinone or epilachnene, see remarks peak E.

G: RT = 9.63, m/z 343.2268, $C_{22}H_{30}O_3$. The database produces 13 isomers for this molecular formula with the identification as 6-methylenetestosterone acetate as one of the more likely considering the retention time as well as the purpose of this product.

H: RT = 10.28, m/z 496.3398, $C_{24}H_{50}NO_7P$. Palmitoyl lyso-lectithin.

With all the identifications listed above it should be kept in mind that these are merely tentative identifications, an indication that a certain compound, in this case androgens and androgen esters, may be present in the sample. To confirm the identity of these compounds a further analyses is required using standard reference compounds. However, the results show that unknown identification without primary reference standards is feasible using UPLC/TOFMS analysis in combination with data processing and accurate mass database searching. For the analysis of the sport supplements this resulted in the tentative identification of several androgens, including methylboldenone (Dianabol) and testosterone, and the esters methyltestosterone propionate or testosterone isobutyrate, testosterone buciclate and methylenetestosterone acetate. In addition, a number of norcodeine- or morphine-like compounds are possibly present in these samples.

# 4 Conclusions

In this study bioassay directed fractionation combined with UPLC/TOFMS identification was tested as a method to detect and identify unknown androgenic compounds. Samples tested comprised of a series of “cold cases”, feed supplements suspected to contain growth promoting substances and already analyzed in the past, however, without detecting any such compounds. A second set of samples consisted of herbs and sport supplements of which it was unknown whether they contained any compounds with androgenic activity but which were meant to improve performance in sports. The results of the androgen bioassay tests on “cold cases” showed that none of the “cold-case” samples contained compounds with androgenic activity. Not only the direct androgen bioassay showed no response but also the tests for pro-androgens, androgen esters and conjugated androgens and pro-androgens did not show positive results indicating the presence of compounds that can metabolically be converted into androgens. Two of the four herbal mixtures tested positive, one for the presence of an androgen, the second for the presence of an androgen ester. While one of the sport supplements tested negative, the results for the others where inconclusive, e.g. indicating the presence of androgen-like compounds, or they reacted toxic in bioassay tests.

Bioassay guided fractionation of the extracts of the positive herbal mixtures resulted in the isolation of the chromatographic fractions contain the compounds responsible for the positive bioassay response. Analysis of these fractions using UPLC/TOFMS resulted in the tentative identification of testosterone phenylacetate (or the isomeric nortestosterone phenylpropionate) and testosterone hexahydrobenzoate in one of the samples and a methyltestosterone isomer in another sample. Using a confirmation method the identity of the first testosterone ester was confirmed as nortestosteron phenylpropionate with an estimated concentration of 0.2 mg/kg product. The identity of testosterone hexahydrobenzoate could not be confirmed since no standard was available. Using GC/MS analysis and comparison of full scan mass spectra the identity of the methyltestosterone isomer was confirmed as 17α-methyltestosterone while it's concentration in the herbal mixture was estimated to be 4 mg/kg product.

For compound identification without primary reference standards an accurate mass database containing approximately 40,000 compounds was developed based on the PubChem database on the internet. Coupling of this database with the TOFMS software and processing of UPLC/TOFMS data files allowed for reversed searching of unknowns in the accurate mass database enabling automatic identification of peaks in a chromatogram. For the analysis of the sport supplements this resulted in the tentative identification of several androgens, including methylboldenone (Dianabol) and testosterone, and the esters methyltestosterone propionate or testosterone isobutyrate, testosterone buciclate and methylenetestosterone acetate. In addition, a number of norcodeine- or morphine-like compounds are possibly present in these samples. The results show that the procedure of bioassay guided fractionation in combination with UPLC/TOFMS analysis and a accurate mass database is very useful for the detection and identification of unknown androgenic compounds in feed and sport supplements. Based on the bioassay response several androgens and especially androgen esters were detected and, in the case of the herbal mixtures, could be isolated using bioassay guided fractionation. The combination of UPLC/TOFMS analysis with an accurate mass database proved valuable for the identification of unknown compounds especially it allows the tentative identification of unknowns even without primary reference standards.

# 5 Recommendations

During the study a few promising techniques have been used and the combination of these techniques enabled the identification of a number of androgens and pro-androgens. However, it was also noticed that the bioassay tests, hoped for to produce a simple "yes/no" response, do not always respond that straightforward. On a few occasions the sample reacted toxic in the bioassay, which is believed to result from the antimicrobial properties of many herbs. Since it is unknown which compounds in different herbal mixtures cause the toxic effects, the only way to eliminate this problem is to dilute the herbal extracts prior to the bioassay test. In practice it was noted that toxic reactions are correlated with the smell of herbal extracts. Extracts having a strong smell more often show toxic effects so this could be used as a rough guide to select those extracts that should be diluted. Dilution, however, will affect the detection limit of the bioassay. It is therefore recommended to determine whether or not extract dilution is effective in avoiding toxic results, which dilution factor should be used, and what that means for the detection limit of the bioassay. Though not really a problem, the sometimes low and difficult to interpret bioassay results seem to indicate the presence of a pro-androgen, androgen ester or other compound that after chemical conversions may result in an androgen. While it is believed that enzymatic hydrolysis and deconjugation will convert most androgen esters en glycosides into the corresponding androgen, this is less clear for pro-androgens. The addition of an S9-mix was selected to convert these into androgens and while this is tested for dehydroepiandrosterone (DHEA), a pro-testosterone, the conversion may not work for other pro-androgens. It therefore would be advisable to test other pro-androgen if these are available. In addition a sample showing such a low response could be tested in a more elaborate way than the conversions listed in Table 1 in this study.

The accurate mass database proved to be a valuable tool for the identification of compounds based on accurate mass. While a Windows Access stand alone version of the database was prepared from the original Windows Excel file, it can only search for $[M+H]^+$ and $[M-H]^-$ ions. Adduct ions like $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$ and $[M+HCO_2]^-$ are not incorporated but could be added fairly easily making the database more comprehensive. In addition the compounds originally to the database should perhaps be reconsidered. Compounds containing sodium (Na) were at the time excluded but since this also excluded a number of sodium salts of organic acids it should be reconsidered. To improve the quality of the identification additional criteria such as isotopic pattern and especially retention time indices could be added to the database. Finally but not least, manual identification of unknowns in UPLC/TOFMS data is a laborious process because of the enormous amount of data and on-line coupling of the database with the instrument software enabling automatic identification of peaks in the chromatogram looks very promising. In this study the accurate mass database was coupled to the TOFMS software (Brukers Target Analyses) allowing automatic identification but also introducing new difficulties. The configuration used in this study is a "reverse search" routine, i.e. the chromatogram is searched for all compounds in the database which is very time-consuming. A forward search, searching the database for peaks of interest in the chromatogram is much more efficient but only possible if the “peaks of interest” can be selected in the chromatogram. In fact, this study is an example of such a forward search routine where the selection of the "peaks of interest" was guided by the bioassay, but in the more general case of "identification of unknowns" such a bioassay guided process is not available and an alternative peak selection process should be developed.

Finally, the results of the combination of UPLC/TOFMS with data processing and an accurate mass database as it was used for identifying compounds without primary reference standards, showed very promising results for the development of a method for the identification on unknowns and is recommended for further development. For the development of a standard operating procedure coupling to TOFMS or OrbitrapMS instruments is required including the development of a forward search routine. For the latter the development of data analysis software allowing the setting of minimum peak heights or areas for peak selection can be an alternative for the bioassay used in this study. In that way it is possible to “tune” the sensitivity of the detection method which can be beneficial depending on the knowledge of the sample under investigation. The introduction of additional criteria such as isotopic pattern and especially retention time indices should limit the number of false positives, in addition to improved data pre-processing routines, for instance by analyzing and subtracting a method blank or even a blank sample if this is available.

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