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(54) **Title:** METHOD FOR EXTRACTING A NATURAL MIXTURE OF CONJUGATED EQUINE ESTROGENS THAT IS DE-
PLETED OF NON-CONJUGATED LIPOPHILIC COMPOUNDS

(54) **Bezeichnung:** VERFAHREN ZUR GEWINNUNG EINES AN NICHT-KONJUGIERTEN LIPOPHILEN VERBINDUNGEN
ABGEREICHERTEN NATÜRLICHEN GEMISCHES KONJUGIERTER EQUINER ÖSTROGENE

(57) **Abstract:** The invention relates to a method for extracting an extract, which contains the natural mixture of conjugated equine es-
trogens, by the liquid-liquid extraction of the mixture of conjugated equine estrogens. The extracted mixture is depleted of non-conju-
gated lipophilic compounds from the group consisting of non-conjugated flavonoids, non-conjugated isoflavonoids, non-conjugated
norisoprenoids, non-conjugated steroids, particularly androstane and pregnane steroids, and comparable non-conjugated compounds.

(57) **Zusammenfassung:** Es wird ein Verfahren zur Gewinnung eines, das natürliche Gemisch konjugierter equiner Östrogene ent-
haltenden Extraktes durch Flüssig-Flüssig-Extraktion des Gemisches konjugierter equiner Östrogene beschrieben, wobei das gewon-
nene Gemisch abgereichert ist an nicht-konjugierten lipophilen Verbindungen aus der Gruppe umfassend nicht-konjugierte Flavono-
ide, nicht-konjugierte Isoflavonoide, nicht-konjugierte Norisoprenoide, nicht-konjugierte Steroide, insbesondere Androstan- und
Pregnan-Steroide, und vergleichbare nicht-konjugierte Verbindungen.

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Method for extracting a natural mixture of conjugated equine estrogens that is
5 depleted of non-conjugated lipophilic compounds

Description

10 The present invention relates to obtaining a natural mixture of conjugated equine oestrogens which is depleted in non-conjugated lipophilic compounds from the group comprising non-conjugated flavonoids, non-conjugated isoflavonoids, non-conjugated norisoprenoids, non-conjugated steroids, in particular androstane and pregnane steroids, and comparable non-conjugated compounds.

15 Oestrogens are used in medicine for hormone replacement therapy. In particular, oestrogen mixtures are used for the treatment and prophylaxis of the disorders of the climacteric period which occur in women after natural or artificial menopause. In this case, natural mixtures of conjugated oestrogens such as are found in the urine of pregnant
20 mares, hereafter referred to as natural mixtures of conjugated equine oestrogens, have proved particularly effective and readily compatible.

The dissolved solids content in the urine of pregnant mares (= pregnant mares' urine, abbreviated hereafter as "PMU") can naturally fluctuate within wide ranges, and
25 may generally lie in a range of 40 to 90 g dry matter per litre. In addition to urea and other usual urine contents, phenolic constituents are contained in the solids content of the PMU in quantities of about 2 to 5% by weight relative to the dry matter. These phenolic constituents include cresols and dihydro-3,4-bis[(3-hydroxyphenyl)methyl]-2(3H)-furanone, known as HPMF. These may be present in free or conjugated form. The PMU contains a
30 natural mixture of oestrogens which is largely present in conjugated form, e.g. as sulphuric acid semi-ester sodium salt (abbreviated hereafter as "sulphate salt"). The content of conjugated oestrogens (calculated as oestrogen sulphate salt) may be between 0.1 and 1% by weight, relative to the dry matter. In addition, further lipophilic compounds may be present in the solids content of the PMU, the quantities of which compounds can fluctuate
35 within wide ranges and cannot be predicted. These lipophilic compounds originate

predominantly from the plants ingested as food by the pregnant mares and comprise above all various flavonoid, isoflavonoid and norisoprenoid derivatives and comparable compounds, such as for example formononetin, genistein, daidzein, biochanin A, equol and coumestrol. These lipophilic compounds originally of plant origin may be present in the urine in conjugated or in free (non-conjugated) form. The lipophilic constituents furthermore occurring in the solids content of the PMU also include non-conjugated steroid derivatives; of these in particular the androstane and pregnane steroids, but also non-conjugated oestrogen derivatives should be mentioned.

Extracts containing natural mixtures of conjugated oestrogens are usually obtained either by means of a solid-phase extraction method or by a method based on various liquid-liquid extraction steps with organic solvents which are not miscible, or only slightly miscible, with water. Generally speaking, in order to be able to be used as active substance constituent for pharmaceuticals, the natural mixture of conjugated oestrogens which is obtained must meet certain pharmaceutical specifications, for example meet the specification laid down in the USP (United States Pharmacopeia) or European Pharmacopoeia. For example, certain limit values must be observed with regard to the content of conjugated oestrogens relative to the dry matter.

US Patents No. 2,551,205 and No. 2,429,398 describe a process for the preparation of a water-soluble oestrogen preparation from PMU, in which initially an aqueous concentrate is obtained by adsorption on activated carbon or other suitable adsorber materials, elution with a water-miscible organic solvent, such as pyridine, and subsequent removal of the solvent, which concentrate contains the major part of the water-soluble oestrogen constituents of the PMU originally used. Whereas in US Patent No. 2,429,398 the concentrate is further purified by extraction with benzene and/or ether, US Patent No. 2,551,205 discloses acidulating the concentrate to a pH value of between 2 and 6, preferably between 4 and 5, and then rapidly extracting it with a organic solvent which is only slightly miscible with water from the group of aliphatic, aromatic or alicyclic hydrocarbons (e.g. hexane, benzene, toluene, cyclohexane) or the chlorinated hydrocarbons (e.g. chloroform, ethylene dichloride, trichloroethylene, carbon tetrachloride, chlorobenzene), in order to separate off undesirable substances such as fats, oils, free phenolic constituents and the non-conjugated steroids by transferring into the organic phase. Finally, the aqueous phase is stabilised by neutralisation. US Patent No. 2,551,205 recommends further purifying the extract obtained by subsequent extraction steps and

precipitation operations. Overall, after performing the method described in US Patent No. 2,551,205 a yield of only about 80% of the oestrogen constituents of the concentrate used is obtained.

5 US Patent No. 2,565,115 describes the extraction of the conjugated oestrogens from PMU with acetone. No statement is made about the purity of the resulting oestrogen fraction.

10 US Patent No. 2,696,265 describes a method in which initially the oestrogens are extracted with an aliphatic alcohol or ketone, such as hexanol, cyclohexanol or cyclohexanone. The oestrogens pass into the organic phase and are then further purified; inter alia, an aqueous phase containing the oestrogens is set to a pH value of 4 with hydrochloric acid and extracted with ethylene dichloride.

15 US Patent No. 2,834,712 discloses a method for the preparation of oestrogen mixtures of significant purity and low toxicity which is based on a large number of individual extraction steps with different solvents and the setting of different pH values. In that method, large volumes of solvents such as hexane and benzene are used. Thus for example in one step an already purified concentrate is dissolved in water, set with
20 hydrochloric acid to a pH value of approximately 5.0 and extracted with benzene and then with ether, in order to separate off the phenolic constituents.

International patent application WO 01/27134 describes a comparatively simple method of extracting conjugated oestrogens from PMU: after the addition of a salt, such
25 as sodium chloride, the PMU is extracted with at least the same volume percent of an organic solvent, such as ethyl acetate, whereupon the conjugated oestrogens pass into the organic phase. The organic phase is separated off and dried in order to obtain the extract. No statements are made in WO 01/27134 about the purity of the extract of conjugated oestrogens which is obtained.

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With the liquid-liquid-extraction method described above and known from the prior art, however, a number of problems occur, such as vigorous foaming, sediment formation, emulsification and poor phase separation. Generally several extraction steps are required, which results in losses and only partial obtention of the oestrogen content. Furthermore,
35 these extraction methods require large volumes of solvents, some of which are harmful to

health. Furthermore, in the patent specifications listed above no statements are made either about the content of non-conjugated lipophilic constituents, such as for example non-conjugated flavonoid, isoflavonoid and norisoprenoid derivatives and comparable non-conjugated compounds, or also non-conjugated steroids, in particular androstane and pregnane steroids, in the products obtained, or about separation of these constituents. These methods known from the prior art either do not provide satisfactory results with regard to the yield or with regard to the purity of the extract obtained, measured on the total hormone content obtained relative to the dry matter, or they are based on a large number of different method steps and the use of large volumes of organic solvents, some of which are undesirable even from a toxicological point of view.

Furthermore, various solid-phase-extraction methods are known from the prior art for obtaining a natural mixture of conjugated equine oestrogens largely depleted in phenolic urine contents. Thus international patent application WO 98/08526 describes a method with which a largely cresol- and HPMF-free mixture which is depleted in phenolic urine contents and contains the natural oestrogen content of the PMU practically completely can be obtained in a solid-phase extraction on a semipolar, in particular non-ionic semipolar, polymeric adsorption resin. International patent application WO 98/08525 describes a similar method in which silica gel is used as adsorber material in the solid-phase extraction. Also Chinese patent application CN 1308083 describes a comparable method in which polar adsorption resins containing cyano groups are used. The extracts obtained are suitable as starting material for the preparation of pharmaceuticals which contain the natural mixture of conjugated oestrogens from PMU as active substance constituent.

The pharmaceutical specification requirements laid down, for example the limit values to be observed with regard to the content of conjugated oestrogens relative to the dry matter, are normally met by the mixtures of conjugated oestrogens obtained from PMU in accordance with the method of WO 98/08526 or the method of WO 98/08525. It has however turned out that in addition to the desired content of conjugated oestrogens also non-conjugated lipophilic compounds may be contained in the dry matter obtained. The non-conjugated lipophilic compounds include for example various non-conjugated flavonoid, isoflavonoid and norisoprenoid derivatives and comparable non-conjugated compounds, such as for example formononetin, genistein, daidzein, biochanin A, equol and coumestrol, but also non-conjugated steroids, in particular androstane and pregnane

steroids, and non-conjugated oestrogens; this list should not be regarded as definitive. The presence of the non-conjugated lipophilic compounds in the mixture of conjugated oestrogens obtained from the PMU cannot be standardised, but both the content and the composition of the free and conjugated lipophilic compounds varies for example according to the food ingested by the pregnant mares.

Although the composition of the natural mixture of conjugated equine oestrogens does not change due to the additional presence of the non-conjugated lipophilic compounds, the content of the conjugated equine oestrogens relative to the dry matter can be reduced. A higher concentration of the active substances, i.e. the conjugated equine oestrogens, in the extract obtained could be achieved by deliberate separation of the non-conjugated lipophilic constituents. Also for reasons of medicament safety it may be useful to separate off the non-conjugated lipophilic compounds in order to ensure a uniform composition of individual extract batches, since in this way the non-conjugated lipophilic constituents, the content and composition of which in the PMU can vary according to the seasonally changing type of food ingested by the pregnant mares, can be eliminated, and thus the resulting extracts would all have a comparable content of conjugated equine oestrogens relative to the dry matter. Furthermore, separation of the non-conjugated lipophilic compounds may be advantageous in order to obtain a uniform physiological spectrum of action. For example, it may be useful to separate off possibly present, non-conjugated lipophilic compounds, which may possibly themselves have an undesirable physiological effect, from the natural mixture of conjugated equine oestrogens.

It is therefore an object of the present invention to develop a technically and economically optimum method for obtaining a natural mixture of conjugated equine oestrogens, the mixture of non-conjugated lipophilic compounds, in particular of non-conjugated flavonoid, isoflavonoid and norisoprenoid derivatives, being largely depleted. In particular, it is the object of the present invention to develop such a method in which only small quantities of solvent which is not harmful to health are used. Furthermore, a method should be developed which is based on only a few method steps and yields an extract of conjugated equine oestrogens which has a comparatively high content of conjugated oestrogens relative to the dry matter. Furthermore, it should be possible, using the method of the present invention, in a simple manner to treat a mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine

which may contain changing and possibly elevated quantities of non-conjugated lipophilic compounds uh that the natural mixture of conjugated equine oestrogens obtained with the present invention has good active substance contents and meets the required pharmaceutical specifications; in particular, it should observe
5 the necessary limit values with regard to the content of conjugated oestrogens relative to the dry matter.

A method has now been devised with which, in a surprisingly simple manner, a mixture of conjugated equine oestrogens can also be obtained from a changing PMU having possibly elevated quantities of non-conjugated lipophilic
10 compounds, the mixture of conjugated equine oestrogens obtained being largely depleted in non-conjugated lipophilic compounds, in particular non-conjugated flavonoid, isoflavonoid and norisoprenoid derivatives. In particular, the method according to the invention can be applied to a mixture of conjugated oestrogens from pregnant mares' urine already depleted in phenolic urine contents, so that
15 with the method a mixture of conjugated equine oestrogens is obtained which has a high product quality and reliably meets the requirements of the pharmaceutical specification, in particular also with regard to the limit values to be observed with regard to the content of conjugated oestrogens relative to the dry matter.

According to a first aspect of the invention there is provided a method for
20 obtaining a mixture of conjugated equine oestrogens, wherein the mixture obtained is depleted in non-conjugated lipophilic compounds selected from the group consisting of: non-conjugated flavonoids, non-conjugated isoflavonoids, non-conjugated norisoprenoids, non-conjugated steroids, in particular androstane and pregnane steroids, and comparable non-conjugated compounds, and that the
25 method is distinguished in that

- a) an aqueous initial phase, selected from the group consisting of:
 - (i) an aqueous solution of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine;

- (ii) an aqueous concentrate of a natural mixture, already depleted in phenolic urine contents, of conjugated estrogens from pregnant mares' urine;
- (iii) a concentrate of a pregnant mares' urine liquid obtained by concentration; and
- (iv) a pregnant mares' urine concentrate, freed from solids and mucilaginous substances by separation processes selected from the group consisting of decantation, separation, filtration and combinations of: any of the before-mentioned processes, or a concentrated urine retentate obtained by membrane filtration, is subjected to a liquid-liquid extraction with a sufficient quantity of an extracting agent and subsequently the aqueous phase is separated off, the extracting agent representing an organic solvent suitable for the extraction of non-conjugated lipophilic compounds of the above group, and which is not miscible, or only slightly miscible, with water, and being selected from the group consisting of straight-chain, branched or cyclic C₄-C₁₀ alcohols, C₂-C₁₀ esterified acids, C₃-C₁₀ aldehydes, C₄-C₁₀ ketones, C₂-C₁₀ ethers, C₃-C₆ nitriles and C₁-C₃ haloalkanes, and mixtures of the aforementioned solvents; and
- b) optionally method step (a) is repeated with the resulting aqueous phase; and
- c) an aqueous phase containing the natural mixture of conjugated estrogens is obtained and optionally concentrated.

The method according to the invention for obtaining a natural mixture of conjugated equine oestrogens is characterised in that the mixture obtained is depleted in non-conjugated lipophilic compounds from the group comprising non-conjugated flavonoids, non-conjugated isoflavonoids, non-conjugated norisoprenoids, non-conjugated steroids, in particular androstane and pregnane steroids, and comparable non-conjugated compounds, and that the method is distinguished in that

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- a) an aqueous initial phase, selected from the group comprising
 - (i) an aqueous solution of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine,
 - (ii) an aqueous concentrate of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine,
 - (iii) a concentrate of a urine liquid, and
 - (iv) a urine concentrate, optionally pre-purified by filtration,

is subjected to a liquid-liquid extraction with a sufficient quantity of an extracting agent which represents an organic solvent suitable for the extraction of non-conjugated lipophilic compounds from the above group, and which is not

miscible, or only slightly miscible, with water, and subsequently the aqueous phase is separated off, and

- b) optionally method step (a) is repeated with the resulting aqueous phase, and
 - c) an aqueous phase containing the natural mixture of conjugated oestrogens is
- 5 obtained and optionally concentrated.

For the method according to the invention an aqueous solution (i) of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine, or an aqueous concentrate (ii) of a natural mixture, already

10 depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine may be used as aqueous initial phase. This aqueous solution or this aqueous concentrate may be obtained by a method such as has already been described for example in international patent applications WO 98/08526 and WO 98/08525 or in Chinese patent application CN 1308083 and is thus familiar to the person skilled in the art from these

15 published patent applications. The contents of WO 98/08526, WO 98/08525 and CN 1308083 are also made the subject of the present application for the purposes of the disclosure. An aqueous solution or an aqueous concentrate of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine may also be the product of a liquid-liquid extraction process, such as described for

20 example in international patent application WO 01/27134. The aqueous solutions or concentrates obtained according to the methods described in the above patent applications can be further concentrated before their use in the method according to the invention in known manner, such as for example by distillation, in order to obtain a concentrate largely freed of organic solvent.

25 Furthermore a concentrate (iii) obtained from the PMU by concentration or a concentrate (iv) obtained from the PMU, which has already been pre-purified by filtration or comparable methods, may be used as aqueous initial phase for the method according to the invention. The collected urine (PMU) is first freed in known manner from mucilaginous substances and solids. Expediently, solids and mucilaginous substances are

30 allowed to settle and are then separated off using known separation methods, for example decanting, separation and/or filtering. Thus the PMU may be passed for example through a known separating means, e.g. a separator, a filtration unit or a sedimenter. A sand bed for example may serve as separating means, or commercially available separators may

35 be used, e.g. nozzle or chamber separators. If desired, a microfiltration unit or an

ultrafiltration unit may also be used, and if these are used it is possible to achieve a largely bacteria-free and virus-free filtered PMU at the same time.

If desired, preservatives, germicides, bactericides and/or anthelmintics may be
5 added to the urine or the urine concentrate.

A concentrated PMU retentate which can be obtained from the PMU by known membrane filtration can also be used as pre-purified urine concentrate (iv). The solids content of the retentate and the composition thereof may vary depending on the PMU
10 used and the membrane used for the membrane filtration, for example the pore width thereof, and also the conditions of filtration. For example, when using a nanofiltration membrane virtually loss-free concentration of the oestrogen content in the PMU retentate can be achieved while simultaneously removing up to 50% by weight of the low-molecular PMU contents. PMU retentates which have been concentrated up to a ratio of
15 approximately 1:10, for example a ratio of approximately 1:7, and the volume of which can thus be reduced to approximately 1/10, for example approximately 1/7, of the original PMU volume can be used for the method according to the invention.

If the concentrate used as aqueous initial phase is a reduced concentrate of the
20 PMU or a PMU concentrate for example already pre-purified by membrane filtration, then the mixture of conjugated equine oestrogens obtained, which has been depleted in non-conjugated lipophilic compounds by the method according to the invention, may already have sufficient purity, but may possibly still contain significant quantities of phenolic urine contents, which have to be separated off by further method steps. Thus with the aqueous
25 phase obtained for example a method can be performed such as has been described in international patent applications WO 98/08526 and WO 98/08525 or in Chinese patent application CN 1308083 and is thus familiar to the person skilled in the art from these published patent applications, in order to obtain a product which also meets the necessary pharmaceutical specifications for conjugated oestrogens with regard to the content of
30 phenolic urine contents.

Within the context of this invention, the use of an aqueous solution of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine, or an aqueous concentrate of a natural mixture, already depleted

in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine as aqueous initial phase is regarded as being particularly preferable.

According to the invention, in method step (a) with the aqueous initial phase
5 described further above a liquid-liquid extraction is carried out with a sufficient quantity of an extracting agent which represents an organic solvent which is not miscible, or only slightly miscible, with water and is suitable for the extraction of non-conjugated lipophilic compounds, in particular of non-conjugated flavonoids, non-conjugated isoflavonoids, non-conjugated norisoprenoids and non-conjugated steroids. Furthermore, the organic
10 solvent should not be miscible, or only slightly miscible, with the aqueous initial phase, "only slightly miscible" meaning that at most 6% by volume dissolved organic solvent is present in the aqueous phase. In principle, any organic solvent which is not miscible with water can be used for this liquid-liquid extraction step, as long as it extracts the non-conjugated lipophilic compounds from the aqueous phase. Suitable examples for the
15 extraction of non-conjugated lipophilic compounds, in particular of non-conjugated flavonoids, non-conjugated isoflavonoids, non-conjugated norisoprenoids and non-conjugated steroids, are the following organic solvents with 1 to 10 C atoms, which may be arranged in a straight-chain, branched or cyclic configuration: C₄-C₁₀ alcohols (such as for example butanol, hexanol, cyclohexanol and pentanol), C₂-C₁₀ esterified acids (such as
20 for example ethyl acetate, methyl acetate, propyl acetate, isopropyl acetate, butyl acetate, amyl acetate, ethyl methyl malonate, dimethyl phosphonate), C₃-C₁₀ aldehydes and C₄-C₁₀ ketones (such as for example butanone, pentanone, hexane-2,4-dione, hexanedial, cyclohexanecarbaldehyde, methyl phenyl ketone and similar), or generally C₃-C₁₀ alkoxy compounds, C₂-C₁₀ ethers (diethyl ether, methyl tert.-butyl ether), C₃-C₆ nitriles, and C₁-C₃
25 haloalkanes (methylene chloride), and also mixtures of the aforementioned solvents. In particular, C₁-C₄-alkyl acetates, hexanol, diethyl ether, methylene chloride, methyl tert.-butyl ether and mixtures of the aforementioned solvents can be used as extracting agents in the present invention. Of this selection, C₁-C₄-alkyl acetates, and in particular ethyl acetate, represent the extracting agent of the present invention which is preferably used.

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In the liquid-liquid extraction with a sufficient quantity of an extracting agent performed according to the invention in method step (a), the volume ratio of the aqueous initial phase to the extracting agent should be understood to be non-limiting in the context of this invention. Generally, a volume of organic solvent is used which corresponds to the
35 volume of the aqueous initial phase, but the ratio of aqueous to organic phase may lie

within a range between 10:1 and 1:10. Preferably, the volume ratio of the aqueous initial phase to the organic extracting agent lies in the range of 5:1 to 1:3; in particular a volume ratio in the range of 2:1 to 1:2 is regarded as expedient.

5 The person skilled in the art will know how to perform such an extraction process from the prior art. Usually a liquid-liquid extraction is performed in an apparatus which permits continuous thorough mixing of the aqueous phase and the organic phase which is not miscible with water. For example, what is called a mixer-settler apparatus, in which the two phases are mixed by stirring, is suitable for performing such an extraction process.

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In principle, the liquid-liquid extraction described according to the invention can be carried out at any pH value of the aqueous initial phase. In a particularly preferred variant of the liquid-liquid extraction described according to the invention, in method step (a) initially the pH value of the aqueous initial phase is set to a value in the range between 4
15 and 12. Preferably, the pH value is set to a value in the range of 4.0 to 7.0, i.e. in the weakly acidic to neutral range. Very particularly preferably, the pH value is set to a value in the range of 4.0 to 6.0, in particular in the range of 4.7 to 5.3.

During the setting of the pH value, the solution initially introduced is expediently
20 mixed thoroughly in a sufficiently large, inert container, such as for example a high-grade steel vat, with a stirrer or a comparable device, in order thus to ensure rapid and uniform setting of the pH value. Conventional bases or acids may be used to set the pH value. Thus for example one of the conventional inorganic or organic acids, expediently a dilute acid, can be used to lower the pH value. For example, the use of dilute sulphuric acid,
25 preferably 1 N sulphuric acid, dilute acetic acid, dilute phosphoric acid or dilute hydrochloric acid, preferably 1 N hydrochloric acid, has proved particularly suitable for lowering or setting a pH value of less than 7.

The liquid-liquid extraction described according to the invention does not require a
30 specific temperature to be set, but can be performed within a wide temperature range, which may be between 5°C and the boiling point of the organic solvent or at most 95°C. Preferably, the liquid-liquid-extraction according to the invention is performed at room temperature, since the additional energy demand is then lowest. Usually ambient temperature is regarded as room temperature; for example, a temperature of between 10°
35 and 30°C is thus designated.

The duration of such a liquid-liquid extraction is regarded as not being limiting in the context of this invention, and may be between 5 min and several hours; the duration will fluctuate according to the quantity of aqueous initial phase used. Typically, the aqueous phase from method step (a) and the organic extracting agent are mixed together for 5 to 60 min, preferably for 10 to 20 min, in order to achieve as complete as possible transfer of the non-conjugated lipophilic constituents from the aqueous phase into the organic phase.

Following the step of extraction by thorough mixing, the phase mixture is allowed to stand, in order to achieve separation of the phases. The phase separation may take from 10 min up to several hours, depending on the volumes used, but preferably the phases are left to stand for 30 to 120 min. When the aqueous phase and the organic phase have separated from each other, the aqueous phase is separated off and retained for re-use, while the organic phase is discarded.

The method step (a) described above may optionally be repeated; this is then followed according to the invention by an optional method step (b), in which with the aqueous phase obtained from method step (a) a liquid-liquid extraction is performed once again with a sufficient quantity of an extracting agent, which represents an organic solvent suitable for the extraction of non-conjugated lipophilic compounds, in particular of non-conjugated flavonoids, non-conjugated isoflavonoids, non-conjugated norisoprenoids and non-conjugated steroids, and which is not miscible, or only slightly miscible, with water.

The possibilities listed above under method step (a) should be regarded as examples for the selection of the extracting agent and the manner of performing the extraction, i.e. the apparatus used, the duration and the temperature of the extraction process.

According to the invention, either different extracting agents or the same extracting agent can be used in both method steps (a) and (b). Preferably the same extracting agent is used in both extraction steps. In particular, in both extraction steps C₁-C₄-alkyl acetates, but in particular ethyl acetate, should be used as extracting agent.

In the liquid-liquid extraction with a sufficient quantity of an extracting agent performed according to the invention in method step (b), the volume ratio of the aqueous phase from method step (a) which has already been extracted once and contains the conjugated equine oestrogens to the extracting agent should be understood as non-limiting in the context of this invention. Generally, a volume of organic solvent is used which is clearly below the volume of the aqueous phase obtained from method step (a), but the ratio of aqueous to organic phase may lie within a range between 40:1 and 1:2. Preferably, the volume ratio of the aqueous phase obtained from method step (b) to the organic extracting agent lies in the range of 20:1 to 1:1; in particular a volume ratio in the range of 10:1 to 2:1 is regarded as expedient.

Following the step of extraction by thorough mixing in method step (b), the phase mixture is allowed to stand, in order to achieve separation of the phases. The phase separation may take from 10 min up to several hours, but preferably the phases are left to stand for 20 to 90 min. When the aqueous phase and the organic phase have separated from each other, the aqueous phase is separated off and retained for re-use, while the organic phase is discarded.

After the separation of the organic phase from the aqueous phase, in method step (c) an aqueous phase containing the natural mixture of conjugated oestrogens is obtained. This aqueous phase contains the natural mixture of conjugated oestrogens occurring in the PMU in addition to only an extremely small proportion of the content of non-conjugated lipophilic constituents originally present in the PMU or the prepared PMU concentrates. If desired, this aqueous phase can be concentrated further in known manner, in order to obtain a concentrate largely freed of organic solvent which is suitable for further processing. Thus for example the still-present residues of organic solvent can be distilled off from the resulting aqueous phase. The distillation means that the dry matter content of the aqueous extract phase can also be set to a concrete value, preferably to a dry matter content in the range between 5 and 15%, in particular to a dry matter content of 9%. Following this, to stabilise the natural mixture of conjugated equine oestrogens obtained, the pH value of the aqueous extract solution can be set to a value in the alkaline range, preferably in the range between 8 and 13, preferably to a value between 9 and 12. Bases usually used for setting the pH value, for example 1N NaOH or Na₂CO₃, are suitable for setting the pH value.

The aqueous phase obtained according to the invention in method step (c), which has optionally been still further worked up or concentrated can serve as the starting material for the preparation of medicaments containing the natural mixture of conjugated equine oestrogens. If desired, an eluent-free solids mixture can be produced by a suitable drying process, such as spray-drying. If the natural mixture of conjugated oestrogens is to be used for the production of solid medicaments, it may be expedient to admix a solid excipient to the aqueous phase containing the conjugated oestrogens already before concentration or drying, in order thus to obtain a solids mixture containing the conjugated oestrogens and excipients. Both the aqueous phase containing the oestrogen mixture and a concentrate or dried solids product prepared therefrom can be processed in known manner into solid or liquid galenic preparations such as for example tablets, coated tablets, capsules or emulsions. These galenic formulations can be prepared by methods known per se using conventional solid or liquid excipients, e.g. starch, cellulose, lactose or talcum or liquid paraffins, and/or using conventional pharmaceutical auxiliaries, for example tablet disintegrants, solubilisers or preservatives. Thus the product containing the conjugated oestrogens can be mixed with the pharmaceutical excipients and auxiliaries in known manner and the mixture converted into a suitable dosage form.

In the liquid-liquid extraction described according to the invention, a large number of non-conjugated lipophilic compounds can be removed simply from an aqueous initial phase which may represent either an aqueous solution of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine, an aqueous concentrate of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine, a concentrate of a urine liquid, or a urine concentrate which has optionally been pre-purified by filtration. The non-conjugated lipophilic compounds which are separated off are in particular non-conjugated flavonoids, non-conjugated isoflavonoids, non-conjugated norisoprenoids and non-conjugated steroids, in this case in particular non-conjugated androstane and non-conjugated pregnane derivatives.

Compared with the conventional liquid-liquid extraction methods, here smaller volumes of organic solvents are used, since a concentrate of the original PMU is always used as aqueous initial phase. If for example an aqueous concentrate which has been obtained by the method described in international patent application WO 98/08526 is used for the aqueous initial phase of the liquid-liquid extraction described according to the

invention, instead of 5000 l PMU only approx. 35 l concentrate and correspondingly small quantities of organic solvents are used for the extraction.

If an aqueous solution of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine, or an aqueous concentrate of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine is used as aqueous initial phase for the liquid-liquid extraction according to the invention, the natural mixture of conjugated equine oestrogens obtained as active substance extract, which is depleted in non-conjugated lipophilic constituents and phenolic urine contents, is distinguished by clear optimisation of the pharmaceutical specification, as was established according to the invention. In particular an 8 to 20% improvement in the ratio of the conjugated equine oestrogens to the dry matter occurs due to the liquid-liquid extraction, without significant losses of conjugated equine oestrogens being observed during the extraction process. Thus the aqueous phase obtained in method step (c), which contains the natural mixture of conjugated equine oestrogens, compared with the prior art has an advantageous composition and a total hormone content which is increased relative to the dry matter. By means of this, a quality product is obtained which is distinctly improved e.g. in relation to the composition and the active substance content.

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It must be regarded as distinctly surprising that a supposedly simple method of liquid-liquid extraction of an aqueous solution or concentrate of a natural mixture, which is depleted in phenolic urine contents, of conjugated oestrogens from the pregnant mares' urine containing different and changing quantities of non-conjugated lipophilic constituents contributes in such a way to improving the quality of the active substance extract obtained. In particular, it is very surprising that the proportion of non-conjugated lipophilic compounds, which can fluctuate greatly both in terms of quantity and composition according to the PMU used, can be reduced by the method according to the invention so reliably that in method step (c) a mixture of natural conjugated equine oestrogens can be obtained as aqueous phase which meets the stringent requirements for pharmaceutical specification, for example the requirements drawn up in accordance with the USP or the European Pharmacopoeia.

Furthermore, it must be regarded as very surprising that when performing the extraction method according to the invention also those non-conjugated lipophilic

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compounds, such as for example defoaming agents, which had previously been added to the PMU as auxiliaries in preceding processing steps, for example in the preparation of the concentrate, are separated off.

5 It has proved a further advantage of the method according to the invention that the further processing of the extracted concentrate obtained, in the form of a natural mixture, depleted in non-conjugated lipophilic compounds, of conjugated equine oestrogens and its conversion into a galenic form is substantially simplified and facilitated. Surprisingly, the active substance extract obtained with the method according to the invention is
10 distinguished by very good drying behaviour and the solids obtained after drying by an extremely good flow ability. Thus for example the extracted concentrate obtained according to the invention can be applied considerably more easily to support material than a non-extracted solution. Also the setting of the active substance concentration becomes simplified and reproducible.

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 The method according to the invention, as already described above in detail, offers a number of advantages and improvements compared with the prior art. Thus the invention makes it possible also to use PMU containing changing quantities of non-conjugated lipophilic constituents, which may for example have an elevated proportion of
20 free flavonoids, free isoflavonoids, free norisoprenoids or free steroid derivatives, in this case in particular of free androstane or pregnane steroids, without the risk of not observing pharmaceutical specifications. With the method according to the invention, a uniform composition of individual extract batches can be ensured, since the non-conjugated lipophilic constituents, the content and composition of which in the PMU may
25 vary according to the type of food ingested by the pregnant mares, are always eliminated and thus the extracts obtained all have a comparable content of conjugated equine oestrogens relative to the dry matter. Furthermore, the separation of the non-conjugated lipophilic constituents achieved with the method according to the invention achieves a higher concentration of the active substances, i.e. the conjugated equine oestrogens, in
30 the extract obtained. The method according to the invention additionally also has economic advantages, since the risk of losing valuable active substances if the pharmaceutical specification is not observed, for example in the case of contents of conjugated oestrogens relative to dry matter which are not sufficient, is considerably reduced. Furthermore, the application of the method described according to the invention
35 permits substantially more accurate and reproducible setting of the active substance

content of the extract obtained. The method according to the invention provides an improved-quality active substance constituent with an increased hormone content relative to the dry-matter content. This active substance constituent is excellently suitable for the preparation of pharmaceuticals which contain a mixture of natural conjugated equine

5 oestrogens as active substance.

The following examples are intended to explain the invention further, but without limiting its scope.

Examples

In the following examples, a general operating procedure is given for obtaining active substance extracts from PMU which contain the natural mixture of the conjugated oestrogens contained in the PMU and are largely depleted in non-conjugated lipophilic compounds, such as for example non-conjugated flavonoids, non-conjugated isoflavonoids, non-conjugated norisoprenoids, non-conjugated steroids, in particular androstane and pregnane steroids, and comparable non-conjugated compounds. It is demonstrated how a quality extract with high active substance contents can be obtained according to the invention even from PMU which may have changing or elevated proportions of non-conjugated lipophilic compounds.

Extraction of an aqueous concentrate of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine

35.3 kg (Example 1) or 26.7 kg (Example 2) of aqueous concentrate of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine is used as aqueous initial phase, which was prepared with the aid of the method described in international patent application WO 98/08526 from approx. 5000 l PMU (dry matter content (= DM) and also contents, determined by means of HPLC and GC, of conjugated oestrogens, for example oestrone sulphate salt, and non-conjugated lipophilic compounds, for example formononetin, are shown in the following table of examples for different batches of aqueous concentrate used). This aqueous concentrate is thoroughly mixed in a high-grade steel vat with the aid of a stirrer, while the pH value is set to a value of approximately 5.0 with 1N H₂SO₄. Ethyl acetate (EA - for quantities see following table of examples) is added to the solution obtained in a mixer-settler apparatus in a ratio of 10:8 aqueous phase to organic phase and the mixture is stirred vigorously for approximately 15 min. Thereafter, the mixture is allowed to stand for approx. 90 min to separate the phases. Following this, the phases are separated and ethyl acetate (EA - for quantities see following table of examples) is added to the aqueous phase again in a ratio of 10:2 aqueous phase to organic phase and the mixture stirred for 15 min. After the extraction, the mixture is left to stand for approximately 30 min to separate the phases. After the separation of the organic phase, the aqueous phase is transferred into a reaction vessel. Any residues of ethyl acetate still present are distilled off from the aqueous phase under normal pressure. As a result of the distillation, the dry matter content is set to

approx. 9%. Following this, the pH value of the solution is set to approximately 11.0 by addition of 1N NaOH or Na₂CO₃. The content of conjugated oestrogens of the aqueous extract phase thus obtained is examined by means of HPLC and GC analysis (dry matter content (= DM) and also contents, determined by means of HPLC and GC, of conjugated oestrogens, for example oestrone sulphate salt, and non-conjugated lipophilic compounds, for example formononetin, are shown in the following table of examples for various batches of aqueous concentrate used). The ratio of the individual hormone constituents to one another, i.e. the relative proportion of oestrone sulphate, equilin sulphate and 17-alpha-DH-equilin sulphate in the mixture of conjugated oestrogens, may vary in the individual batches owing to the natural fluctuations in the PMU. The desired ratio of the individual hormone constituents relative to each other can be set by deliberate mixing together of individual batches.

	Example 1			Example 2		
	Content [mg/g]	Quantity [g]	% by weight DM	Content [mg/g]	Quantity [g]	% by weight DM
Aqueous initial concentrate		35,300			26,700	
Dry matter (% by weight)	(9.8)	3,495		(9.4)	2,510	
Conjugated oestrogens (total)	26.2	924	26.7	25.8	688	27.4
Oestrone sulphate	12.2	431	12.4	16.9	452	18.0
Equilin sulphate	9.3	328	9.5	5.8	156	6.2
17-alpha-DH-equilin sulphate	4.7	166	4.8	3.0	80	3.2
Formononetin	0.17	5.9	0.17	0.32	8.4	0.34
1. EE extraction (acetic ester)		25,400			19,400	
2. EE extraction (acetic ester)		6,400			4,900	
Aqueous extract phase obtained		34,000			23,500	
Dry matter (% by weight)	(8.9)	3,026		(8.9)	2,092	
Conjugated oestrogens (total)	26.3	896	29.6	27.9	656	31.4
Oestrone sulphate	12.4	420	13.9	18.5	434	20.8
Equilin sulphate	9.3	317	10.5	6.3	147	7.0
17-alpha-DH-equilin sulphate	4.6	158	5.2	3.2	75	3.6
Formononetin	0	0	0	0	0	0

This extraction method can be performed analogously if instead of the aqueous concentrate of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine as aqueous initial phase either a concentrate of a urine liquid, a urine concentrate, optionally pre-purified by filtration, or an aqueous solution of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine, is used as aqueous initial phase.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for obtaining a mixture of conjugated equine oestrogens, wherein the mixture obtained is depleted in non-conjugated lipophilic compounds selected from the group consisting of: non-conjugated flavonoids, non-conjugated isoflavonoids, non-conjugated norisoprenoids, non-conjugated steroids, in particular androstane and pregnane steroids, and comparable non-conjugated compounds, and that the method is distinguished in that

a) an aqueous initial phase, selected from the group consisting of:

(i) an aqueous solution of a natural mixture, already depleted in phenolic urine contents, of conjugated oestrogens from pregnant mares' urine;

(ii) an aqueous concentrate of a natural mixture, already depleted in phenolic urine contents, of conjugated estrogens from pregnant mares' urine;

(iii) a concentrate of a pregnant mares' urine liquid obtained by concentration; and

(iv) a pregnant mares' urine concentrate, freed from solids and mucilaginous substances by separation processes selected from the group consisting of decantation, separation, filtration and combinations of: any of the before-mentioned processes, or a concentrated urine retentate obtained by membrane filtration,

is subjected to a liquid-liquid extraction with a sufficient quantity of an extracting agent and subsequently the aqueous phase is separated off, the extracting agent representing an organic solvent suitable for the extraction of non-conjugated lipophilic compounds of the above group, and which is not miscible, or only slightly miscible, with water, and being selected from the group consisting of straight-chain, branched or cyclic C₄-C₁₀ alcohols, C₂-C₁₀ esterified acids, C₃-C₁₀ aldehydes, C₄-C₁₀ ketones, C₂-C₁₀ ethers, C₃-C₆ nitriles and C₁-C₃ haloalkanes, and mixtures of the aforementioned solvents; and

- b) optionally method step (a) is repeated with the resulting aqueous phase; and
- c) an aqueous phase containing the natural mixture of conjugated estrogens is obtained and optionally concentrated.

5 2. The method according to claim 1, wherein an aqueous solution (i) of a
natural mixture, already depleted in phenolic urine contents, of conjugated
oestrogens from pregnant mares' urine, or an aqueous concentrate (ii) of a
natural mixture, already depleted in phenolic urine contents, of conjugated
10 oestrogens from pregnant mares' urine is used as aqueous initial phase in
method step (a).

3. The method according to claim 2, wherein the depletion in phenolic urine
contents occurs by solid-phase extraction on non-ionic semipolar adsorption
resins or on hydropobized silica gel.

15 4. The method according to any one of claims 1 to 3, wherein the extracting
agent is selected from the group consisting of C₁-C₄-alkyl acetates, hexanol,
diethyl ether, methylene chloride, methyl tert.-butyl ether and mixtures of the
aforementioned solvents.

5. The method according to claim 4, wherein the extracting agent is C₁-C₄-
alkyl acetate, preferably ethyl acetate.

20 6. The method according to any one of claims 1 to 5, wherein in method step
(a) the aqueous initial phase is set to a pH value in the range between 4 to 12.

7. The method according to claim 6, wherein the pH value is set in the range
of 4.0 to 7.0, in particular in the range of 4.0 to 6.0, and preferably in the range of
4.7 to 5.3.

25 8. The method according to any one of claims 1 to 7, wherein in method step
(a) the volume ratio of the aqueous initial phase to the extracting agent lies in the
range of 5:1 to 1:3, preferably in the range of 2:1 to 1:2.

9. The method according to any one of claims 1 to 8, wherein in method step (b) the volume ratio of the aqueous initial phase obtained from step (a) to the extracting agent lies in the range of 20:1 to 1:1, preferably in the range of 10:1 to 2:1.
- 5 10. A method for obtaining a natural mixture of conjugated equine oestrogens, said method substantially as hereinbefore described with reference to the Examples.

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