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(54) Title: DETECTION AND DETERMINATION OF PHENOL-LIKE SUBSTANCES BY MEANS OF DIRECT WET-CHEMICAL METHODS

(54) Bezeichnung: NACHWEIS UND BESTIMMUNG VON PHENOLARTIGEN INHALTSSTOFFEN MITTELS DIREKTER NASS-CHEMISCHER METHODEN

(57) Abstract

The invention relates to modified wet-chemical methods of analysis for detecting and determining phenol-like compounds and cannabinoids by means of colour reactions in a hitherto unknown and simple manner, and directly in fresh plants, plant materials and preparations thereof. These methods allow for the selective, specific wet-chemical detection of individual cannabinoids by means of special colour reactions. The methods are suitable for use in rapid analysis test kits and enable even non-specialists to carry out the intended determinations simply and quickly.

(57) Zusammenfassung

Die Erfindung betrifft modifizierte nasschemische Analysemethoden zum Nachweis und zur Bestimmung von phenolartigen Verbindungen und Cannabinoiden mittels Farbreaktionen auf bisher nicht bekannte, einfache Weise, direkt in Frischpflanzen, Pflanzmaterialien und deren Zubereitungen. Mit diesen Verfahren kann der spezifische, nasschemische Nachweis einzelner Cannabinoide mittels spezieller Farbreaktionen selektiv durchgeführt werden. Die Verfahren sind für die Anwendung in Analysen-Schnelltest-Packungen einsetzbar und die vorgesehenen Bestimmungen können auch für Laien schnell und problemlos durchgeführt werden.

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**Detection and determination of phenol-like constituents
by means of direct wet-chemical methods**

Description

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Phenol-like compounds are important, pharmacologically active constituents of various plants and preparations produced therefrom.

10 Cannabinoids in particular, the principal active compounds of the hemp plant (*Cannabis sativa*), have recently been in the focal point of scientific interest due to their varied and valuable pharmacological properties.

15 Various physical, chemical and also various wet-chemical methods are known for their detection. The latter are mainly used in the form of spot tests using spray reagents.

20 US 3,656,906 describes the detection of cannabinoids in biological materials such as blood plasmе [sic] and urine by condensation of the previously extracted cannabinoids with polycarboxylic acid and subsequent photometric determination.

25 In US 4,816,415, the detection of cannabinoids in body fluids is likewise described. In this process, the cannabinoids are bound to arylcarboxylic acids with the aid of a special filter funnel and the cannabinoid content is then determined by colour reaction with known reagents, namely Fast Blue salt BB, Fast Bordeaux salt Gp, Fast Green salt, Duquenois-Negm reagent or
30 Beam reagent.

In EP 132 313 A2, cannabinoid detection methods for drug test packs based on diazonium reagents are described. The reaction is carried out on filter paper and the reagents are applied in liquid form or as a
35 spray.

These and similar processes are relatively troublesome to carry out and preparation of the sample material by measures such as drying, extraction, filtration and evaporation is usually necessary in

order to be able to carry out the corresponding determinations.

This requires a considerable expenditure of time and effort and can occasionally lead to a change in the substance composition of the sample material. Moreover, the use of toxic or highly corrosive chemicals is often necessary in these methods, which is problematical for wide use by the lay person.

In the area of the search for drugs, the wet-chemical Duquenois reaction in the form of a reagent kit is mainly employed for the rapid recognition of cannabis. However, this method is less specific, cannot give any information at all on the actual cannabinoid ratio, works with concentrated acid and is moreover not very simple to carry out.

The object was thus to make available a process which makes possible a simple and rapid detection of cannabinoids, which can easily be carried out on the spot by laypersons.

This object is achieved by a process for the wet-chemical detection of phenol-like substances, in particular of cannabinoids and materials containing substances of this type, characterized in that the sample material is mixed directly with a colour-forming reagent A, an additional reagent B is added for development of the specific colour reaction and the phenol-like substance to be determined is determined and, if appropriate, also quantitatively detected by measurement of the colour formed.

The sample is mixed directly, i.e. for example in pretreated or untreated form, also without prior drying, directly [sic] with a colour-forming reagent A. The reagent A can be employed as a solution of a colour-forming substance with or without addition of organic solvents. Preferred examples of the colour-forming substance are Fast Black K, Fast Blue salt B, 2,6-dibromoquinone chlorimide, 2,6-dichloroquinone chlorimide, formaldehyde, acetaldehyde, salicylaldehyde, vanillin, p-dimethylaminobenzaldehyde,

p-diethylaminobenzaldehyde, iron(III) chloride, 4-aminoantipyrine, 4-aminophenol or potassium hexacyanoferrate. The reagents are preferably introduced in the form of 0.001 to 10% strength solutions of the colour-forming substance (weight/volume) in water and/or primary, secondary and tertiary alcohols having 1 to 10 carbon atoms, with or without addition of organic solvents such as saturated and unsaturated hydrocarbons, halogenated hydrocarbons, ethers, ketones, carboxylic acid esters and/or aromatic hydrocarbons. Reagent B is preferably a solution of a colour-developing substance, e.g. of a base such as, for example, an alkali metal hydroxide and/or an alkali metal carbonate, an ammonium or alkali metal salt of an organic acid which is optionally substituted by one or more organic radicals, e.g. alkyl groups, or mixtures thereof and is present in the form of a solution of 1 to 50% of the colour-developing substance (wt/vol.) in water and/or primary, secondary and tertiary C₁-C₁₀ alcohols or mixtures thereof. If appropriate, ammonium or alkali metal salts of organic acids such as, for example, acetic acid, propionic acid, butyric acid, oxalic acid, fumaric acid, malic acid, sorbic acid, benzoic acid, salicylic acid, phthalic acid, phenylacetic acid, naphthylacetic acid and/or indole-3-acetic acid can be added to reagent B, e.g. in amounts of 0.1 to 20% (wt/vol.).

Surprisingly, it was possible to develop a process in which specifically selected colour reactions are modified such that the reagents are added immediately to the solvent functioning as an extracting agent and are employed directly in the wet-chemical detection reaction. As a result, the pretreatments of the sample material which are otherwise necessary, such as drying, extraction and final evaporation, are no longer necessary.

In the process according to the invention, it is achieved by suitable choice and design of the reagents, and also by specific modification during the

implementation of the process, that the phenolic substances predominating in each case are made visible to the eye by development of a specific, clearly differentiable colour shade and thus can be detected directly by wet-chemical means in a previously unknown, simple and rapid manner.

By refinement of the process, it was additionally possible to achieve an optimum, particularly easily readable graduation, and a stability of the resulting colour shades lasting for a number of hours.

In this way, it is for the first time also possible to subject fresh, untreated sample material and even fresh plants in pretreated or untreated form directly to the investigation even without prior drying, e.g. in the field, and to analyse for phenolic or cannabinoid constituents.

The process according to the invention has proved particularly suitable in the detection of cannabinoids, the main active compounds of hemp which are of pharmacological interest. Thus, for the first time a differentiation between drug and industrial hemp is possible in young or adult, male or female plants from an age of two weeks. In addition, the determination of the degree of maturity of hemp plants can even be performed directly on fresh plants in a rapid and simple manner.

The areas of use of the process according to the invention include agricultural hemp cultivation (industry), hemp-growing, hobby gardening (home grow area), cannabis research and medicine, cannabis consumption (quality control) and official uses (drug control). The process can furthermore be employed for the detection of cannabis preparations in biological sample materials, in particular human samples, e.g. body fluids such as blood, saliva or urine, or tissue samples such as hair or nails.

It was possible by means of specific modifications of the reagents and the processes to find

novel methods for the selective wet-chemical detection of individual specific cannabinoids.

The processes can also be employed for the identification and quality assessment of ethereal oils and plant materials by determination of phenolic substances present. As a result of the simple procedure and the use of reagents and solvents which are as non-toxic as possible, it is also possible for inexperienced lay persons to carry out the described tests without difficulties.

On account of the simple design of the process and of the reagents necessary therefor, these processes are particularly suitable for analysis packs (test kits) which can be employed directly there and then. By means of photometric measurement of the main absorption maxima of the developed colour shades, which is known to the person skilled in the art, the processes can also be used for the quantitative determination of the detected phenolic substances. The solvents used in reagent solution A are preferably mono- or polyhydric alcohols on their own or mixtures thereof, which on the one hand guarantee optimal extraction of the active compound and on the other hand, however, also guarantee good dissolving properties for the reagent used and an interference-free colour reaction. The reagent solutions B needed for colour development were designed such that they can be added to the reagent mixture only in drop quantities for the purpose of handling which is as simple and effective as possible. As a result of the addition of salts of organic acids to reagent solution B, it was additionally possible to achieve better readability of the developed colour shades, and an improved shelf life thereof.

The invention is intended to be explained and illustrated with the aid of the following examples, but without being restricted to these.

1. Wet-chemical determination of phenols and cannabinoids in various materials and fresh plants

a) Reagents:

- 5 Reagent A: Solution of 0.01% p-aminophenol in isopropanol
Reagent B: 10% strength solution of sodium hydroxide in H₂O-isopropanol (2:1)

10 b) Procedure:

20 to 100 milligrams of the sample material are mixed with 2 ml of reagent A in a clear, colourless test tube, shaken several times, treated with 2 drops of reagent B, shaken again and the resulting
15 colouration is read off after 10 minutes.

c) Colour reactions

20 I. Blank value → yellowish-grey (negative)

II. Fresh hemp plants

Immature drug hemp → violet-reddish
Matured drug hemp → blue-green
Mature EU-industrial hemp (Felina 34) → violet-reddish

25 III. Ethereal oils

Rosemary oil → olive-green
Oregano oil → blue
Thyme oil → blue

30 IV. Phenolic compounds

Thymol → deep-blue
Cannabidiol (CBD) → violet-pink
Tetrahydrocannabinol [sic] (THC) → green-blue
35 Cannabinol (CBN) → blue

2. Selective wet-chemical detection of tetrahydrocannabinol (THC)

a) Reagents

Reagent A: 0.15% iron(III) chloride in absolute ethanol

Reagent B: 1% ammonium acetate in absolute ethanol

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b) Procedure:

10 to 100 mg of the sample material are mixed with 1.5 ml of reagent A and shaken in a clear, colourless sample tube. The mixture is then treated with 5 drops of reagent B. The colour shades are read off after 1 minute.

c) Colour reactions:

Blank value → weakly brownish-yellow = negative
15 Cannabidiol → (weakly brownish-yellow) = negative
Cannabinol → (weakly brownish-yellow) = negative
THF → orange red-brown = positive

3. Selective wet-chemical detection of THC

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a) Reagents:

Reagent A: 0.01% strength solution of Fast Black K in ethanol

25 Reagent B: 5% strength sodium hydroxide solution in ethanol, 50% strength

b) Procedure:

30 50 to 100 mg of sample material are mixed with 5 ml of reagent A in a clear, uncoloured sample tube and thoroughly shaken several times. One drop of reagent B is added, the mixture is thoroughly shaken once more and the resulting colouration is read off within 1 minute.

c) Colour reactions:

Blank value = pale violet-pink = negative
Cannabidiol = pale violet-pink = negative
Cannabinol = pale violet-pink = negative
5 THC = brownish orange-red = positive

4. Selective wet-chemical detection of cannabinol

a) Reagents:

10 Reagent A: 0.01% strength solution of 2,6-dichloro-quinone chlorimide in isopropanol
Reagent B: aqueous, saturated potassium carbonate solution

15 b) Procedure:

10 to 50 mg of sample are mixed with 5 ml of reagent A in a transparent, uncoloured sample tube and thoroughly shaken several times. The mixture is then treated with 1 drop of reagent B, vigorously shaken for
20 1 minute and the resulting colouration is immediately read off.

c) Colour reactions:

Blank value → pale brownish-yellow colouration
25 = negative
Cannabidiol → pale brownish-yellow colouration
= negative
THC = pale brownish-yellow colouration
= negative
30 Cannabinol = blue colouration = positive

5. Use of the test for cannabinoid determination (for reagents see Example 1)

a) Sample selection

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Type of analysis	Type of sample
Hemp maturity test	female infructescences
Differentiation between industrial and drug hemp	fully developed digitate leaves of male, female or young plants of 2 weeks minimum age
Cannabis preparations quality control	marihuana, hashish, hashish oil, hemp foliage, stems and leaves

10 For the determination of the degree of maturity, fresh, undried plant material is used, since on drying the cannabinoid content can be altered (e.g. conversion of cannabidiol to THC).

15 For the differentiation of industrial or drug hemp, fully formed digitate normal leaves (fresh or dried) of the plant and not the inflorescences or infructescences are used. By means of a test on the normal, digitate leaves of a specific young plant which is at least two weeks old, its maximally achievable future cannabinoid spectrum can be determined.

b) Sample quantities

Sample type	Comment	Fresh	Dried
Plant material			
Female inflorescences or seed-bearing pods	Comminuted without leaves and young shoots	approx. 100 mg (~ 2 knife tips)	approx. 10 mg (small knife tip)
Hemp leaves	fully formed from male or female or young plants	approx. 300 mg (large knife tip)	approx. 50 mg (one knife tip)
Cannabis preparations			
Marihuana	comminuted	-	approx. 10 mg
Hashish	finely comminuted	-	approx. 5 mg (very small knife tip)
Hashish oil	-	-	approx. 2 mg

If the developed colour shades are too intensive and thus difficult to read, the test should be repeated with a reduced quantity of sample. Excessively small quantities of sample can in turn afford colour shades which are pale, indistinct and shifted into the yellow. In this case, the test should be repeated using a larger quantity of sample.

c) Implementation of the tests

A small amount of the samples (see under quantities of sample) is added to a glass tube. The tube is half filled with reagent A, closed with a plastic cap and shaken several times in the course of approximately 1 min.

The tube is then opened and treated with one drop of reagent B, immediately closed again and shaken again. The resulting colouration of the supernatant solution can be read within one minute. The best results are obtained in clear daylight or against a white surface.

: By separation of the coloured, supernatant
solution from the sediment of the sample material by
means of decantation into another glass tube, the
colouration obtained can be protected from light and,
5 with closure, can be kept stable for longer.

d) Colour reaction and meaning

Sample used	yellowish	reddish	olive-brown	green	blue-green	blue
female infructescences	negative	immature drug hemp or mature EU industrial hemp (Felina 34)	half-mature drug hemp	mature drug hemp	completely mature drug hemp	blue overmature drug hemp
normal leaves of young or old, male or female plants	negative	industrial hemp	drug hemp	drug hemp	drug hemp	drug hemp
cannabis preparations	negative	very little THC	little THC	average THC	great deal of THC	old THC + CBN
cannabinoid content	negative	mainly CBO	CBD and THC	mainly THC	great deal of THC	THC and CBN
CBD/THC ratio	negative	> 2:1	2:1	< 2:1	<1:>1	THC and CBN

The lower detection limits achievable using the coloured processes [sic] are 0.04 mg for CBC, 0.02 mg for THC and 0.02 mg for CBN.

Claims

1. Process for the wet-chemical detection of phenol-like substances, in particular of cannabinoids
5 and materials containing substances of this type, characterized in that the sample material is mixed directly with a colour-forming reagent A, an additional reagent B is added for development of the specific colour reaction and the phenol-like substance to be
10 determined is determined and, if appropriate, also quantitatively detected by measurement of the colour formed.

2. Process according to Claim 1, characterized in that, as reagent A, solutions of a
15 colour-forming substance, such as, for example, Fast Black K, Fast Blue salt B, 2,6-dibromoquinone chlorimide, 2,6-dichloroquinone chlorimide, vanillin, salicylaldehyde, formaldehyde, acetaldehyde, p-dimethylaminobenzaldehyde, p-diethylaminobenz-
20 aldehyde, iron(III) chloride, 4-aminophenol or potassium hexacyanoferrate are used.

3. Process according to Claim 1, characterized in that, as reagent B, solutions of a
25 base, such as, for example, an alkali metal hydroxide or an alkali metal carbonate and/or of an optionally substituted ammonium or alkali metal salt of an organic acid or mixtures thereof are used.

4. Process according to one of Claims 1 to 3, characterized in that 0.1 to 20% of an ammonium or
30 alkali metal salt of an organic acid are added to the reagent B.

5. Process according to one of the preceding claims,
35 characterized in that 0.01 to 500 mg of sample material are mixed with 1 to 10 ml of reagent A and, after shaking, with 1 to 10 drops of reagent B in a sample tube and, after repeated shaking, the resulting colouration is read within a predetermined period of time.

6. Process according to one of the preceding claims,

characterized in that the reagent A is made up of a 0.001 to 10% strength solution of a colour-forming substance in water or/and primary, secondary and/or tertiary C₁-C₁₀-alcohols and reagent B is made up of a solution of 10 to 50% alkali metal hydroxide in water or alcohols or mixtures thereof.

7. Process according to one of the preceding claims,

characterized in that the reagent A is made up of a 0.05 to 5% strength solution of iron(III) chloride in water and/or primary, secondary or tertiary C₁-C₁₀-alcohols and reagent B is made up of a solution of 0.1 to 5.0% of an ammonium salt of an organic acid and the resulting colour reaction can be read after approximately 2 minutes.

8. Process according to one of the preceding claims,

characterized in that reagent A is made up of a 0.001 to 5% strength solution of 2,6-dibromoquinone chlorimide in primary, secondary and/or tertiary C₁-C₁₀-alcohols, and reagent B is made up of a saturated aqueous solution of potassium carbonate and the resulting colour shade is read off within approximately 30 seconds.

9. Process according to one of the preceding claims,

characterized in that it is used for the identification and quality determination of cannabis preparations such as marihuana, hashish, hashish oil and other cannabinoid-containing materials, for the determination of the degree of maturity of hemp plants, and for differentiation of drug and industrial hemp in adult male or female and also in young hemp plants in fresh or alternatively dried form.

10. Process according to one of Claims 1 to 8, characterized in that it is used for the detection of cannabis preparations in biological sample materials,

e.g. of body fluids such as blood, saliva or urine, or tissue samples such as hair or nails.

11. Process according to one of Claims 1 to 8, characterized in that it is used for the wet-chemical
5 detection of various phenol-like compounds, in ethereal oils, resinoids, absolutes, concretes, fresh plants, plant drugs, plant extracts and aromatic substances, and for identification and characterization and quality determination thereof.
- 10 12. Reagent kit for carrying out the process according to one of the preceding claims, characterized in that it contains the two reagents A and B bottled separately, e.g. in corrosion-resistant
15 device, and several small sealable clear, colourless sample tubes for carrying out the colour reaction and a colour comparison scale for reading off and evaluating the resulting colour shades.

Abstract

The invention relates to modified wet-chemical analytical methods for the detection and for the
5 determination of phenol-like compounds and cannabinoids by means of colour reactions in a previously unknown, simple manner, directly in fresh plants, plant materials and preparations thereof. Using this process, the specific, wet-chemical detection of individual
10 cannabinoids can be carried out selectively by means of specific colour reactions. The processes can be employed for use in analytical rapid test packs and the intended determinations can also be carried out rapidly and without problems for [sic] lay persons.

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