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## **POSTERS**

# Ginkgo Biloba food supplements, results of a pilot evaluation on Health claims, Quality and Safety

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## Aim of the study

Health claims on functional foods and supplements have to be substantiated since the approval of the EU regulation on nutrition and health claims. Besides, these products have to be safe for the users and composition of these products, especially the content of the active components, should be in accordance with the declaration on the label.

## Methods

Three claimed health effects of Ginkgo Biloba were selected:

1. improved blood circulation;
2. effects on symptoms of old age;
3. improved memory.

Per health effect an overview of publicly available, objective scientific data was made. Next, the PASSCLAIM criteria were applied to assess if the data can support the claims. The quality is evaluated by analyzing the main components of 29 Ginkgo Biloba food supplements by HPLC. To complete the multidisciplinary assessment a "classical" toxicological risk assessment was performed.

## Results

### Health Claims

There is insufficient evidence to prove the 3 health claims of Ginkgo Biloba. The main reason is missing of clinical data of healthy subjects. Most of the studies are related to studies with patients.

These studies prove the positive effects of Ginkgo Biloba in the treatment of peripheral arterial occlusive disease (PAOD).

However this is a *medical* claim.

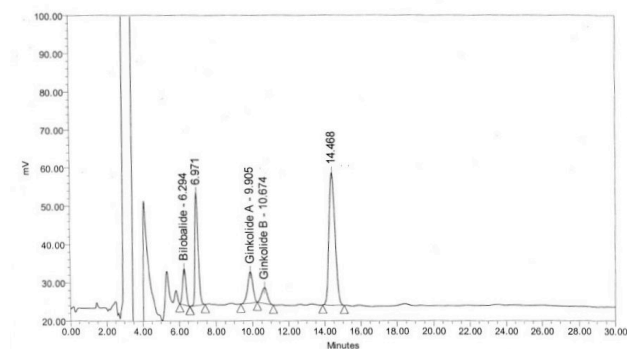


Figure 1. Representative HPLC-chromatogram of the terpene analysis

### Quality

29 Ginkgo Biloba food supplements were analysed for flavonoids and terpenes by HPLC. A licensed medicinal product and a 24%-standardised extract were used as reference.

For 13 products the label claim was unclear. Only 2 products comply with the claimed content (90-110%).



Picture 1. Ginkgo Biloba leaf

To prove the presence of the 24%-standardised extract the ratio of the Bilobalide content and the total Terpene content is calculated. Only 3 products probably contain standardised Ginkgo Biloba 24%- standardised extract based on this ratio.

### Safety

Long term toxicological data is missing, although the Ginkgo Biloba supplements are often used during a life time. Immunotoxic, cytotoxic, carcinogenic and genotoxic properties are attributed to Ginkgolic acids. This substance is limited in the Ginkgo Biloba standardised extract at 5 ppm. However because in most cases it is not clear if the standardised extract is incorporated in the food supplement higher levels of Ginkgolic acid are quite possible.

### Conclusion

The Health claims are not substantiated and the Safety of the Ginkgo Biloba food supplements can not be guaranteed, because in most cases, the standardised 24%-extract is not incorporated in the food supplement.

Based on the quality results, only 10% of the food supplements in this study will be effective.

### References

- www.passclaim.ilsa.org
- Passclaim; Consensus on Criteria, Eur. J. Nutr (2005) [Suppl 1.] 44: 1/1-1/2
- HPLC; Mostafa et al, Il Farmaco 60 (2005)

Commissioned by the Food and Consumer Product Safety Authority (VWA); [www.vwa.nl](http://www.vwa.nl)

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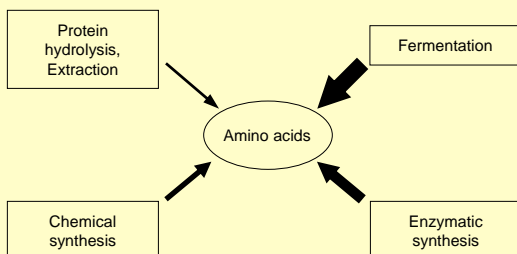
# AMINO ACIDS: PUTATIVE IMPURITIES RESULTING FROM PRODUCTION PATHWAY AND THEIR DETERMINATION

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## Introduction

Currently, in the Ph. Eur. the purity of amino acids is controlled by means of TLC limiting ninhydrin-positive substances to 0.5%. This method allows mainly the determination of other amino acids as impurities arising when amino acids are produced by protein hydrolysis. However, since the sensitivity of TLC is poor, Novatchev developed a method, which is suitable for determination of amino acids at 0.1% level, based on derivatization of amino acids with 9-fluorenylmethyl chloroformate (FMOC) and separation of derivatives by means of micellar electrokinetic chromatography (MEKC) [1]. As can be seen in the following schema, nowadays industrial production of amino acids is dominated by enzymatic synthesis and fermentation.



For this reason, the spectrum of putative impurities has to be broadened towards starting material, intermediates and by-products of biosynthesis. Since 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) has a wide reactivity spectrum towards primary amines it was used as derivatization reagent in combination with MEKC for impurity profiling of amino acid samples [2].

Nevertheless, putative impurities of biotechnologically produced amino acids include carboxylic acids, which were not detectable with one of the methods mentioned. Most of those organic acids show UV absorbance at low wavelengths; however, UV response of most amino acids is poor. Here, we present preliminary results of using evaporative light scattering detection (ELSD) in combination with HPLC separation for determination of organic acids and amino acids simultaneously. In principle, the chromatographic signals obtained with ELSD originate from light scattered by particles which are formed via nebulisation and evaporation of the eluent. Therefore, the signals are not dependant on the presence of chromophoric groups of the substances, but on the concentration of the compounds.

## Experimental

### Analysis of amino acids by means of CE

CE-measurements were performed on a Beckman P/ACE System MDQ (Fullerton, CA, USA), equipped with an LIF-detector with an excitation by argon-ion laser at 488 nm and emission wavelength at 520 nm. The fused-silica capillaries were of 75 µm internal diameter and effective length of 60.0 cm (total length of 70.0 cm).

For derivatization with CBQCA, amino acids were dissolved in phosphate buffer ( $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ , 125 mM, pH 7.0) to give a 0.45 mg/ml solution. Derivatization was performed by mixing 10 µl of the sample solution with 10 µl of CBQCA solution (10 mM in DMSO) and 10 µl of KCN solution (10 mM in water). The mixture was allowed to react for 10 h at ambient temperature. Samples were diluted to 1.0 ml with water and filtered through a 0.22 µm PVDF filter before injection (anodic side, 3.45 kPa, 5 s). Derivatized samples were stored at 2 – 8 °C. Separation was performed at 25 °C using a voltage of 20 kV. The employed separation buffer was a borate buffer (25 mM, pH 9.20), containing 25 mM SDS. The LIF-detector was used for the detection of fluorescent derivatives.

### Analysis of amino acids by means of HPLC-ELSD

HPLC-measurements were performed on an Agilent System 1100 LC (Böblingen, Germany) consisting of a vacuum degasser, a binary pump and an autosampler. The detector was an evaporative light scattering detector Model Sedex 85 (ERC, München, Germany) and was set as follows: drift tube 35 °C unless otherwise indicated, nebulizer gas pressure 3.6 bar, gain 10.

Separation was carried out using a Synergi Hydro-RP column (150 x 4.6 mm; 4 µm) characterised by a polar end-capping. The mobile phase was a filtered and degassed mixture of mobile phase A (0.5% heptafluorobutyric acid in water) and mobile phase B (0.3% heptafluorobutyric acid in methanol) (83:17, V/V). Flow rate was set at 0.8 ml/min.

Model mixtures were prepared by dissolving citric acid, fumaric acid, maleic acid, malic acid, succinic acid, alanine, aspartic acid and glutamic acid at varying concentrations in water. Additionally, 0.2 mg/ml samples of alanine and aspartic acid, respectively, were spiked with putative impurities at a level of 0.01 mg/ml. Samples were filtered through a 0.22 µm filter and 10 µl were injected in the HPLC system.

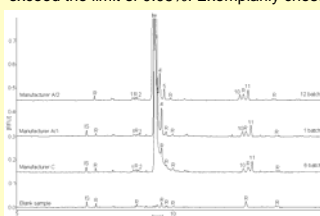
## Results and discussion

### Analysis of amino acids by means of CE

The impurity profile of a number of amino acid samples collected from the European market was determined by means of MEKC after derivatization of the samples with FMOC and CBQCA, respectively.

Although the analysis of isoleucine batches by means of the Ph. Eur. TLC method provided no indication of impurities, after derivatization with FMOC and MEKC separation the detected impurity peaks could be assigned to amino acids. Glycine, alanine, leucine and valine were found to be putative impurities in isoleucine batches [3].

However, an advantage of CBQCA is the possibility to label amino acids, amino sugars and low molecular weight peptides simultaneously. Analysing CBQCA labelled isoleucine batches revealed a number of peaks apart from amino acids indicating the presence of so far unknown impurities. Up to 16 impurities could be detected, but only a few of them exceed the limit of 0.05%. Exemplarily chosen electropherograms are shown below.

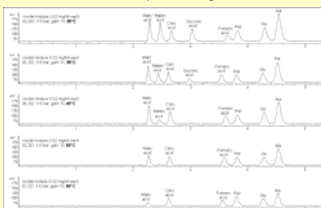


Electropherograms of isoleucine batches (0.45 mg/ml)

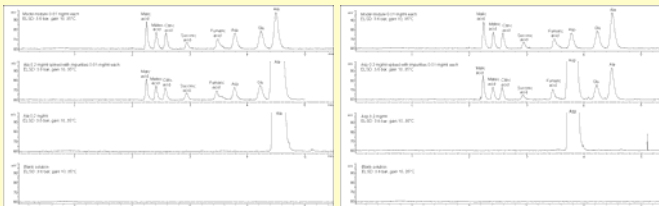
Peak identity:  
(R) Reagent peaks  
(IS) Internal standard: D-(+)-glucosamine

### Analysis of amino acids by means of HPLC-ELSD

Buffer additives for ELSD are limited to volatile substances, e.g., ammonium acetate, formic acid, trifluoroacetic acid. For our purpose, heptafluorobutyric acid being a volatile ion-pair reagent was used. Separation of a model mixture at different evaporator temperatures is shown below. At higher temperatures baseline noise decreased; however, some of the peaks decreased as well or disappeared. In detail, maleic acid and succinic acid are volatile at higher temperature. A temperature of 35 °C was found to balance baseline noise and peak height and was therefore chosen for measurements.



The following figures present alanine and aspartic acid, respectively, spiked with organic acids. Aspartic acid was chosen as it is commonly produced by enzymatic synthesis starting from fumaric acid being a putative impurity. Manufacturing process of alanine include enzymatic decarboxylation of aspartic acid. Therefore, aspartic acid as well as fumaric acid are expected to be impurities of alanine.



## Conclusion

Impurity profiling of amino acids should be considered in view of the production processes since different manufacturing processes result in different impurities. Tests for quality control described in the Ph. Eur. are mainly adapted to amino acids obtained from extraction. Nevertheless, analysing CBQCA labelled amino acids revealed the presence of so far unknown impurities.

For simultaneous detection of amino acids and organic acids being putative impurities HPLC in combination with ELSD was employed. Although separation of compounds of interest was achieved the method is lacking of sensitivity and must be improved in this view.

## Literature

[1] N. Novatchev, U. Holzgrabe; J. Pharm. Biomed. Anal. 26, 779-789, 2001. [2] N. Novatchev, U. Holzgrabe; J. Pharm. Biomed. Anal. 28, 475-486, 2002. [3] S. Kopec, U. Holzgrabe; Electrophoresis 28, 2007, in press.

## Acknowledgement

Thanks are due to the Federal Institute for Drugs and Medical Devices, Bonn, Germany, for financial support.

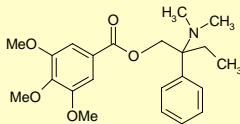
# ELABORATION OF A TEST FOR DETERMINATION OF RELATED SUBSTANCES OF TRIMEBUTINE AND TRIMEBUTINE MALEATE BY MEANS OF HPLC

Susanne Kopec and Ulrike Holzgrabe

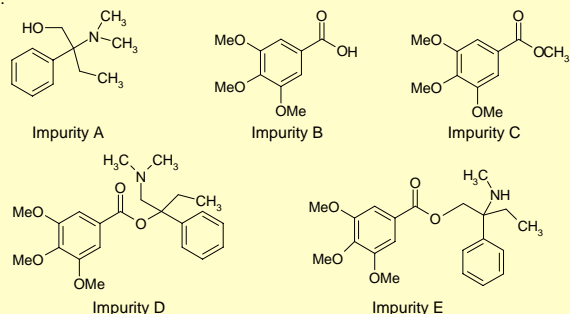
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## Introduction

Due to their antispasmodic actions on the gastrointestinal tract, trimebutine and trimebutine maleate are clinically used for treatment of gastrointestinal disorders such as irritable bowel syndrome [1]. Thus, trimebutine was included in the work program of the Ph. Eur. Commission for elaboration of monographs. Within this study, it was aimed to develop an HPLC method for determination of related substances of trimebutine (TMB) and trimebutine maleate (TMB maleate).



The structural formulae of the known impurities are shown below. Impurity A is a key intermediate in the synthesis of trimebutine and impurity E is the main active metabolite in vivo.



Initially, the ion-pair chromatography method described in the monograph for TMB maleate in the Japanese Pharmacopoeia [2] was adapted. Nevertheless, considering the results for LOD / LOQ of the substances, a second method without using ion-pair reagents was developed. A number of TMB and TMB maleate batches were tested by means of the both methods.

## Experimental

All measurements were performed on an Agilent System 1100 LC (Böblingen, Germany) consisting of a vacuum degasser, a binary pump, an autosampler, a thermostated column compartment and an UV-visible diode array detector.

### Ion-pair chromatography

Separation of TMB and its impurities was achieved employing a Nucleosil C8 column (250 x 4.0 mm; 5 µm) at 30°C. The mobile phase was a filtered and degassed mixture of mobile phase A (8.8 mM of sodium pentane sulfonate added to a 0.01 M perchloric acid solution adjusted with ammonium acetate to pH 3.0) and acetonitrile (80:20, V/V). Flow rate was set at 1.2 ml/min and the detection wavelength at 254 nm.

Samples containing 10 mg/ml of TMB were prepared by dissolving the substance in acetonitrile and TMB maleate in a mixture of acetonitrile and 0.01 M HCl (50:50, V/V), respectively, and filtered through a 0.22 µm filter. 10 µl of the samples were injected in the HPLC system.

### Reversed-phase chromatography

TMB and its impurities were separated on a hydrophilic end-capped C18 stationary phase (Aquasil C18, 125 x 4.6 mm; 5 µm) applying gradient elution with phosphate buffer (30 mM, adjusted with conc. phosphoric acid to pH 3.0) and acetonitrile. The gradient program is shown in the following table:

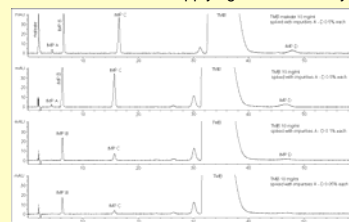
Time [min]	Acetonitrile [%]	Flow rate [ml/min]
0.00	22	1.20
3.00	35	1.20
6.00	35	1.20
6.50	35	0.80
15.00	40	0.80
16.00	40	0.80

Afterwards, the column was equilibrated with 22% acetonitrile for 4 min at 1.20 ml/min. The column was thermostated at 25°C. As the substances show maximum absorbance at approx. 215 nm, this wavelength was chosen for detection of TMB and the impurities. TMB maleate samples were dissolved at 1 mg/ml in the sample solvent (20 mM phosphate buffer pH 2.5 and acetonitrile (80:20, V/V)). Since TMB is sparingly soluble, the substance was dissolved in acetonitrile and diluted with the sample solvent. Samples were filtered through a 0.22 µm filter and 20 µl were injected in the HPLC system.

## Results and discussion

### Ion-pair chromatography

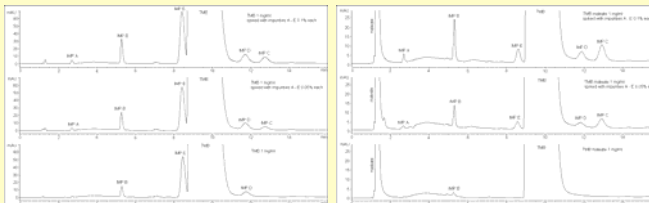
As shown in the figure below, the separation of TMB / TMB maleate and its impurities was achieved within 60 min applying a C8 stationary phase and isocratic elution.



The LOD / LOQ of impurities A and D were found to be critical parameters at the chosen detection wavelength of 254 nm: LOQ of impurity A was determined to be 0.24% and LOQ of impurity D 0.15%, respectively. However, decreasing the detection wavelength was not applicable due to baseline noise at lower values caused by the mobile phase.

### Reversed-phase chromatography

Further experiments showed the suitability of a hydrophilic end-capped C18 column for separation of TMB / TMB maleate and its impurities without using ion-pair reagents.

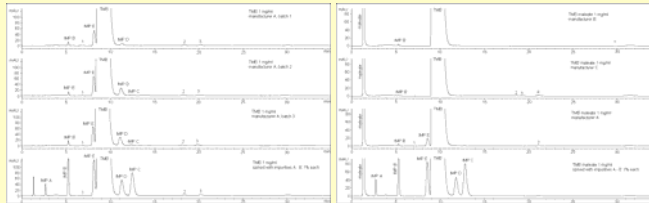


The detection wavelength was decreased from 254 nm to 215 nm resulting in better responses of the substances and, therefore, in an improved LOD / LOQ. The LODs for each compound determined as signal-to-noise ratio (S/N) higher than 3 and the LOQs determined as S/N higher than 10 are summarised in the following table.

IMP	LOD [µg/ml]	LOD [%]	LOQ [µg/ml]	LOQ [%]
A	0.02	0.002	0.13	0.013
B	0.02	0.002	0.05	0.005
C	0.02	0.002	0.11	0.011
D	0.09	0.009	0.26	0.026
E	0.10	0.010	0.20	0.020

Considering these results, the method is suitable for determination of related substances at a level of 0.1% and the disregard limit can be set at 0.05%.

A number of TMB and TMB maleate batches of different manufacturers were analysed. Exemplarily chosen chromatograms are shown below.



Considering the results of batch testing, TMB maleate batches can be divided in two groups: one containing only trace levels of impurity E and one containing impurity E in the range of 0.1% to 0.5%. However impurity E was present in all batches of TMB in a concentration up to 1%.

## Conclusion

Comparing both methods, the reversed-phase chromatography is characterised by a shorter analysis time and a better LOD / LOQ of impurities. Thus, this method was proposed for determination of related substances of TMB / TMB maleate. Nevertheless, critical pairs of the reversed-phase method are the separation between impurity E and the main peak as well as the separation of impurities C and D. Both should be controlled for system suitability testing.

## Literature

[1] J. E. F. Reynolds; Martindale: The Extra Pharmacopoeia; 31. Edition, Royal Pharmaceutical Society, London, 1996. [2] Monograph for trimebutine maleate; Japanese Pharmacopoeia, 1993.

## Acknowledgement

Thanks are due to the Federal Institute for Drugs and Medical Devices, Bonn, Germany, for financial support.

