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# Variation in Vasicine Content and Pharmacognostic Characters of Morphotypes of *Adhatoda zeylanica* Medic.

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**Abstract:** As part of the gene bank activity of CMPR many collections of *Adhatoda zeylanica* Medic. were made from varying agroecological regions in South India. These collections could be grouped into four morphotypes based on growth habits and other morphological characters. These four morphotypes were evaluated for their chemical and pharmacognostical characters and significant differences among the morphotypes were noticed. Among quality characters vasicine content, fingerprint profiles, stomatal index, leaf architecture and venation pattern showed significant variation indicating the predominance of additive gene effects. A reverse phase HPLC method for the quantitative determination of vasicine in the morphotypes was developed based on which variation in the vasicine content was observed. Thin layer chromatographic analysis showed variation in the chemical composition of these morphotypes. These studies indicated variability in the chemical composition and pharmacognostic characters among the morphotypes of *A. zeylanica*. Two of the morphotypes containing significantly higher vasicine indicated the presence of chemical diversity, providing adequate scope for selection of superior chemotypes having high therapeutic value and economic benefit.

**Key words:** *Adhatoda zeylanica*, pharmacognosy, *Vasaka*, vasicine

## INTRODUCTION

*Adhatoda zeylanica* Medic. (Common name-*Vasaka*), a perennial shrub of the family Acanthaceae, is a highly reputed Ayurvedic medicinal plant used in the treatment of cough, bronchitis, asthma, tuberculosis and recommended for other ailments of the respiratory system (Sivarajan and Balachandran, 1994). Leaves, flowers and roots of this plant are used in herbal drugs against cancer (Pandey, 2002). Different methods of isolation and estimation of active constituents from *A. zeylanica* have been suggested (Narayana *et al*., 1995; Johri and Zutchi, 2000; Srivastava *et al*., 2001) and its seasonal variations were also reported (Arambewela *et al*., 1988; Das and Chowdhury, 2005). Bagchi *et al*. (2003) reported that *A. zeylanica* showed higher content of vasicine compared to *A. beddomei* all throughout the year. Work done in various aspects of *A. zeylanica* such as morphology, anatomy, diseases and pests, chemical constituents, propagation, tissue culture, toxicology, intellectual property rights, phytochemical studies, pharmacological studies, medicinal uses, antimicrobial activity, traditional knowledge and quantitative standards have been reviewed by George *et al*. (2006). Raw material of *Vasaka,* consisting mainly of leaves, is very much in demand in the herbal pharmaceutical industry. Plant materials, mostly collected from natural sources, are mostly used in the preparation of the herbal drugs. However, such materials show a wide range of variation in quality. Attempts have been made to cultivate the plant in several parts of India, but so far large-scale cultivation to meet the

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requirements of the pharmaceutical industry has not been started. Variation in the quality of the plant material is observed, which may be due to the seasonal variations of the secondary metabolites and the difference in the agro-ecological conditions. Apart from such factors, other reasons for the difference in quality may include the intra-specific variation, as there exists different morphotypes of *A. zeylanica*.

In the present work comparative phytochemical and pharmacognostic characters of the different morphotypes of *A. zeylanica* collected from South India were studied taxonomically and authenticated and maintained in the germplasm bank of CMPR. The study on the germplasm collection of the morphotypes of *A. zeylanica* led to the grouping of the collections into four morphotypes viz. large type (AZ1), small type (AZ2), spreading type (AZ3) and intermediate type (AZ4). Considering the therapeutic and economic aspects of *A. zeylanica* and its diversity in India, it is essential to assess the extent of diversity at chemical and pharmacognostic levels. Such an analysis can provide scope for the selection of superior genotypes and chemical varieties (chemovars) for future use.

## MATERIALS AND METHODS

### Plant Material

Accessions of *A. zeylanica* were collected from different phytogeographical zones of south India. Based on the macro and micromorphological characters, four distinctly different morphotypes (AZ1, AZ2, AZ3 and AZ4) were identified and subjected to detailed chemical and pharmacognostical studies. Leaves from four accessions belonging to each of the morphotypes maintained in the germplasm bank (Collection No. 01169, 02342, 02340, 02341 respectively), Arya Vaidya Sala, Kottakkal, Kerala, India, were collected in September 2004. The voucher specimens of the plant materials are archived in the Botany Department (Centre for Medicinal Plants Research, Arya Vaidya Sala, Kottakkal, Kerala, India). The plant material was dried at 50°C for 48 h and powdered. The powdered material was stored in glass bottles protected from light and moisture at room temperature.

### Extraction

Powdered leaves (20 g) of each of the morphotypes were mixed thoroughly with 20 mL 10% ammonia solution and then refluxed with ethanol (3×200 mL) for 1 h over water bath at 60°C. The individual extracts were combined, filtered and concentrated to dryness under reduced pressure below 60°C. The concentrate was dissolved in ethanol and used for the analysis.

### TLC Analysis

Thin Layer Chromatographic analyses of the extracts were performed on silica gel 60 F254 TLC plates (10×10 cm; Merck, Darmstadt, Germany). Aliquots (10 µL) of the extracts were applied on the plates as bands. Plates were developed in TLC chamber previously saturated (30 min) with the mobile phase, chloroform: methanol (9:1 v/v). The chromatogram was developed to 80 mm and then dried to remove the solvents. The plates were visualized under UV 254 and 365 nm and then sprayed with Dragendorff's reagent.

### HPLC Analysis

### Standard Preparation

Stock solution of vasicine (Natural Remedies, Bangalore, India) was prepared in ethanol (Analytical Grade; Merck (India) Limited) at 1000 µg $mL^{-1}$.

The HPLC analyses were carried out at 25°C on a Shimadzu (Kyoto, Japan) LC-10AT liquid chromatograph equipped with a Phenomenex Luna (Torrance, CA, USA) $RP_{18}$ column (250×4.60 mm i.d.; 5 µm) with a Phenomenex guard column (4×2 mm i.d.; 5 µm). The samples were injected using a 20 µL loop (Rheodyne Rohnet Park, CA, USA). The mobile phase consisted of methanol: water

(40:60) and the separation was performed by using isocratic elution at a flow rate of 1 mL $min^{-1}$. The chromatograms were run for 35 min. Shimadzu SPD-M10A photodiode array detector was used at 298 nm for detection. The data was processed on a Shimadzu LC Workstation Class-VP System.

**Method Validation Studies**

Samples for validation analyses were obtained by the same procedure as described previously. A vasicine solution in three different concentrations was added to the AZ1 extract to evaluate the method's performance. The method was validated for precision, accuracy, limit of detection and of quantification, linearity range and sensitivity. Intra and inter-day variability measurements were used to determine the method's precision and accuracy. The intra-day precision of four individual samples was examined one day and inter-day precision was determined on five independent days. The intra and inter-day accuracy was determined by the same procedure. Linearity was determined using the vasicine reference standard, at different levels of concentration, each level in triplicate.

In order to determine the accuracy of the method, known amount of vasicine was added in three concentrations (50, 150 and 250 μg $mL^{-1}$) to AZ1 leaves prior to extraction. Recovery rates were calculated by the following equation:

$$R\ (\%) = [\text{Average of the amount of vasicine quantified in the sample}] / [\text{Average of the amount of vasicine added to the sample}] \times 100$$

**Quantitative Analysis of Vasicine**

The total vasicine content was determined by external standard method. The standard solutions were prepared in ethanol (50-250 μg $mL^{-1}$). The vasicine content was found out from the calibration curve of the standard plotted between the concentration and area by calculating the area under curve of the sample.

**Anatomy and Micromorphology**

The leaves of the morphotypes were processed as per the standard procedures for histological studies (Johansen, 1940). Measurements were taken using ocular and stage micrometers. Photomicrographs were taken using Canon digital camera attached to Zeiss microscope.

## RESULTS AND DISCUSSION

Different mobile phases for the separation of *A. zeylanica* extracts were tested, using silica gel TLC plates. The mobile phase that had the best resolution and separation was chloroform: methanol (9:1). The TLC fingerprint profiles of all the four morphotypes of *A. zeylanica* were compared. The extracts were separated into individual components using appropriate solvent systems after performing trials with a wide range of solvents. The solvent system for TLC separation was selected based on the efficiency of separation and degree of resolution of the system. The Rf (Retardation factor) was calculated for identifying the spots. The TLC fingerprint profile comprises of the bands resolved, Rf values and colour/fluorescence when scanned at UV 254 and 365 nm (Table 1). Derivatisation of the TLC plates with Dragendorff's reagent was done and visualized. Among the four morphotypes studied, AZ1 and AZ2 showed more similarity in the TLC profiles. Like wise similarity was observed between AZ3 and AZ4. Overall, the TLC fingerprint profiles showed significant differences, which indicated variation in their chemical constituents.

The HPLC fingerprint profiles of all the four morphotypes of *A. zeylanica* were compared (Fig. 1). Among the four morphotypes, AZ1 and AZ2 showed similar profiles both qualitatively and quantitatively, so also similarity existed between AZ3 and AZ4. The amount of vasicine present in the plant extracts was calculated by external standard method and the morphotypes showed variation

Table 1: TLC fingerprint profile of the four morphotypes of *A. zeylanica*

| | 254 nm | | 365 nm | | Visible light | | DRG reagent | |
|---|---|---|---|---|---|---|---|---|
| Morphotype | Rf | Colour | Rf | Colour | Rf | Colour | Rf | Colour |
| AZ1 | 0.40 | Black | 0.07 | Orange | 0.18 | Green | 0.10 | Brown |
| | 0.49 | Blue | 0.21 | White | 0.31 | Green | 0.55 | Orange |
| | 0.58 | Black | 0.41 | Orange | 0.55 | Green | 0.60 | Orange |
| | 0.75 | Black | 0.48 | White | 0.60 | Yellow | 0.70 | Green |
| | 0.81 | Black | 0.55 | White | 0.71 | Yellow | 0.75 | Green |
| | 0.96 | Black | 0.60 | Blue green | 0.75 | Light Green | 0.81 | Orange |
| | | | 0.69 | Orange | 0.81 | Light Green | 0.86 | Green |
| | | | 0.73 | Blue green | 0.86 | Green | 0.98 | Yellowish |
| | | | 0.82 | Orange | 0.98 | Orange | | Green |
| | | | 0.84 | Blue green | | | | |
| AZ2 | 0.38 | Black | 0.07 | White | 0.18 | Light brown | 0.18 | Orange |
| | 0.48 | Black | 0.21 | White | 0.25 | Light brown | 0.36 | Orange |
| | 0.81 | Blue | 0.41 | White | 0.31 | Light brown | 0.51 | Orange |
| | 0.95 | Black | 0.48 | White | 0.36 | Light brown | 0.52 | Orange |
| | | | 0.55 | Blue green | 0.51 | Green | 0.59 | Green |
| | | | 0.60 | Orange | 0.52 | Light brown | 0.71 | Green |
| | | | 0.72 | Blue green | 0.59 | Green | 0.81 | Green |
| | | | 0.75 | Blue green | 0.71 | Pale yellow | 0.98 | Green |
| | | | 0.80 | Orange | 0.78 | Pale yellow | | |
| | | | 0.83 | Blue green | 0.81 | Green | | |
| | | | | | 0.98 | Orange | | |
| AZ3 | 0.40 | Black | 0.41 | White | 0.25 | Green | 0.18 | Orange |
| | 0.48 | Blue | 0.43 | Orange | 0.31 | Light brown | 0.31 | Orange |
| | 0.81 | Black | 0.44 | Blue green | 0.36 | Light brown | 0.36 | Orange |
| | 0.95 | Black | 0.48 | Blue green | 0.46 | Light brown | 0.46 | Orange |
| | | | 0.55 | Orange | 0.51 | Light brown | 0.51 | Orange |
| | | | 0.59 | Blue green | 0.60 | Green | 0.60 | Green |
| | | | 0.61 | Blue | 0.63 | Light brown | | |
| | | | 0.63 | Blue green | 0.80 | Pale green | | |
| | | | 0.80 | Orange | 0.98 | Green | | |
| | | | 0.85 | Blue green | | | | |
| | | | 0.90 | Orange | | | | |
| AZ4 | 0.39 | Black | 0.41 | White | 0.25 | Green | 0.25 | Green |
| | 0.48 | Blue | 0.43 | Orange | 0.31 | Green | 0.36 | Orange |
| | 0.75 | Black | 0.44 | Blue green | 0.36 | Light brown | 0.36 | Orange |
| | 0.81 | Black | 0.48 | Blue green | 0.41 | Green | 0.41 | Orange |
| | 0.96 | Black | 0.56 | Orange | 0.61 | Green | 0.46 | Orange |
| | | | 0.60 | Blue green | 0.64 | Light brown | 0.52 | Green |
| | | | 0.65 | Blue green | 0.82 | Light green | 0.61 | Green |
| | | | 0.71 | Orange | 0.90 | Green | | |
| | | | 0.85 | Orange | 0.98 | Pale green | | |
| | | | 0.98 | Blue green | | | | |

Table 2: Vasicine content of the four morphotypes of *A. zeylanica*

| Sample | Rt | Vasicine content* % w/w |
|---|---|---|
| Standard Vasicine | 2.3 | - |
| Morphotype AZ1 | 2.3 | 1.41±0.10 |
| Morphotype AZ2 | 2.2 | 1.22±0.04 |
| Morphotype AZ3 | 2.3 | 2.35±0.12 |
| Morphotype AZ4 | 2.3 | 2.57±0.13 |

n = 5, *Mean±SD

in vasicine content. AZ4 (2.57% w/w) and AZ3 (2.35% w/w) contain higher amounts of vasicine compared to AZ1 (1.41% w/w) and AZ2 (1.22% w/w) (Table 2). The reported yield of alkaloids from different samples in India ranged from 0.541-1.105% w/w on dry basis (Anonymous, 2003). The method validation was done by performing precision, accuracy, limit of detection (LOD) and quantification (LOQ), linearity range and sensitivity studies (Table 3).

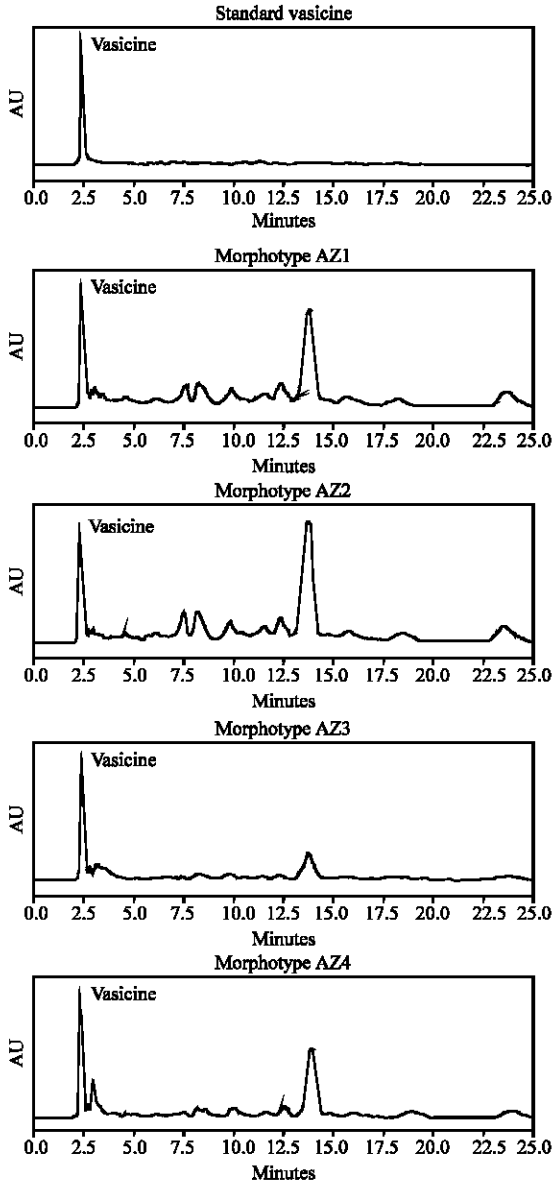
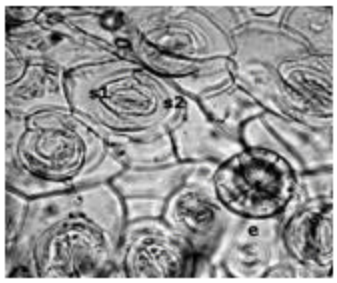
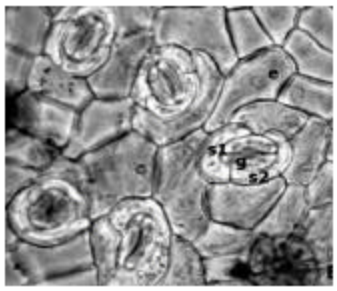



Fig. 1: HPLC Chromatograms of standard vasicine and the four morphotypes of *A. zeylanica*

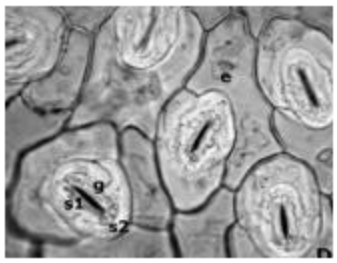
All the four morphotypes of *A. zeylanica* studied, have diacytic stomata and variations were observed in the characters of epidermal and subsidiary cells. The epidermal cells of AZ1 and AZ2 are similar in size and shape, having straight cell walls. The epidermal cells are larger in AZ3 and AZ4, than those of AZ1 and AZ2 with slightly wavy and prominently wavy cell walls respectively (Fig. 2). Highest stomatal index was observed in AZ3, where as AZ1 and AZ2 showed almost equal stomatal index and AZ4 had the lowest. Variation in the number of veins in the midrib region of the morphotypes were noticed; five in AZ1 and AZ2 and three in AZ3 and AZ4. The study also indicated that AZ2 closely resembles AZ1 while the other two morphotypes are distinctly different (Table 4).



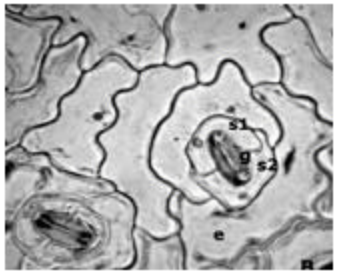

Morphotype $AZ_1$




Morphotype $AZ_2$




Morphotype $AZ_3$




Morphotype $AZ_4$

e – epidermal cells, g – guard cells, s – subsidiary cells

Fig. 2: Stomatal pattern of morphotypes of *A. zeylanica*

Table 3: Method validation parameters for the estimation of vasicine in *A. zeylanica*

| Parameters | Result |
|---|---|
| Instrumental Precision (% CV) (n = 5) | 0.091 |
| Repeatability (%CV) (n = 5) | 1.13 |
| Limit of detection | 200 ng $mL^{-1}$ |
| Limit of quantification | 800 ng $mL^{-1}$ |
| Specificity | Specific |
| Linearity (Correlation coefficient) | 0.0987 |
| Range | 40-400 μg |
| Recovery | 99.10±0.29% |

Table 4: Micro morphology and anatomy of *A. zeylanica* morphotypes

| Leaf architecture and anatomy | AZ1 | AZ2 | AZ3 | AZ4 |
|---|---|---|---|---|
| Shape | | | | |
| Whole lamina | Symmetrical | Symmetrical | Symmetrical | Symmetrical |
| Base only | Slightly asymmetrical | Slightly asymmetrical | Symmetrical | Asymmetrical |
| Form | Elliptic | Ovate | Elliptic | Elliptic |
| Apex | Attenuate | Attenuate | Acuminate | Acuminate |
| Base | Acute, cuneate | Rounded | Cuneate to deccurent | Attenuate |
| Margin | Entire | Entire | Entire | Entire |
| Texture | Membranous | Membranous | Chartaceous | Membranous |
| Glandular position | Laminar | Laminar | Laminar | Laminar |
| Petiole | Normal | Normal | Normal | Slightly Winged |
| Venation | Pinnate Camptodromous Brochidodromous | Pinnate Camptodromous Brochidodromous | Pinnate Camptodromous Brochidodromous | Pinnate Camptodromous Brochidodromous |
| Epidermis | Single layered with uni and multicellular hairs | Single layered uni hairs and multi cellular hairs | Single layered with a few unicellular and multicellualr hairs | Single layered with a few multicellular |
| Mesophyll | Bifacial | Bifacial | Bifacial | Bifacial |
| Mid rib region | Thin walled Collenchymatous patches on both sides | Thin walled | Thin walled | Thick walled |
| No. of veins | Major one and two secondaries on either side | Major one and two secondaries on either side | Major one and single secondary on either side | Major one and single secondary on either side |
| Cystolyth | Present | Present | Present | Present |
| Crystals | Plenty in the midrib region | Plenty in the midrib region | A few in the midrib region | A few in the midrib region |
| Stomatal index* | 19.3±0.23 | 18.2±0.12 | 21.9±0.11 | 15.3±0.10 |

n = 5, *Mean±SD

There were significant differences among the morphotypes for different chemical and pharmacognostical characters. The chemical variation could be characterized from HPLC and TLC fingerprint profiles, vasicine content and presence of compounds. Among the pharmacognostic characters, variations were observed in stomatal index, leaf architecture and venation pattern. The variability in the morphotypes collected from various parts of South India, indicate that they are locally adapted genotypes. Such variations provide adequate scope for selection of chemically superior morphotypes (chemovars). The two morphotypes with high vasicine content are such superior chemotypes that can be further evaluated and recommended for commercial cultivation. Adequate pharmacological evaluation should be done before their use in the manufacture of herbal and ayurvedic formulations.

The small morphotype (AZ2) is often mistakenly treated as *A. beddomei* and the small variety is the one preferred in the Ayurvedic medicine preparation. However this is only a morphological variant and is not the *A. beddomei* described originally by Hooker (1885). This is not in anyway superior to the larger morphotype; in fact it contains lesser amounts of vasicine compared to *A. zeylanica* (Bagchi *et al*., 2003). The original *A. beddomei* is a highly endangered and extremely rare species and is not cultivated at all.

At CMPR further studies on biomass production, yield per unit area and other agrotechnological aspects are in progress. AZ3 seems to be a chemotype that may be useful for herbal drug preparations and thus needs to be promoted and popularized in future.

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## REFERENCES

Anonymous, 2003. The Wealth of India, Raw Materials. Vol. A: NISCAIR, CSIR, New Delhi, pp: 77.

Arambewela, L.S.R., C.K. Ratnayake and J.S. Jayasekera, 1988. Vasicine contents and their seasonal variation in *Adhatoda vasica*. Fitoterapia, 59: 151-153.

Bagchi, G.D., P.D. Dwivedi, F. Haider, S. Singh, S. Srivastava and S.K. Chattopadhyay, 2003. Seasonal variation in vasicine content in *Adhatoda* species grown under North Indian plain conditions. J. Medi Aromatic Plant Sci., 25: 37-40.

Das, C., R. Poi and A. Chowdhury, 2005. HPTLC determination of Vasicine and Vasicinone in *Adhatoda vasica*. Phytochem. Anal., 16: 90-92.

George, S., K.V. Tushar, P.S. Udayan, S.S. Raja, K.P. Unnikrishnan, A.B. Remashree, I. Balachandran and P.N. Ravindran, 2006. *Adhatoda zeylanica*: A review. J. Trop. Med. Plants, 7: 137-147.

Hooker, J.D., 1885. Flora of British India. Reeve and Co., Ltd., London, 4: 540.

Johansen, D.A., 1940. Plant Microtechnique. Mc Graw-Hill, New York.

Johri, R.K. and U. Zutchi, 2000. Mechanism of action of 6, 7, 8, 9, 10, 12-hexahydro azepino-(2, 1-b) quinazolin-12-one-(RLX): A novel bronchodilator. Indian J. Physiol. Pharmacol., 44: 75-81.

Narayana, D.B.A., S. Agarwal, S.K. Luthra and N.S. Srinivas, 1995. A HPLC method for the quantitative analysis of vasicine in *Adhatoda vasica*. Indian Drugs, 32: 583-586.

Pandey, G., 2002. Anticancer Herbal Drugs of India with Special Reference to Ayurveda. Sri Satguru Publications, Delhi.

Sivarajan, V.V. and I. Balachandran, 1994. Ayurvedic Drugs and Their Plant Sources. Oxford and IBH Publishing Co. Pvt. Ltd.

Srivastava, S., R.K. Verma, M.M. Gupta, S.C. Singh and S. Kumar, 2001. HPLC determination of vasicine and vasicinone in *Adhatoda vasica* with photo diode array detection. J. Liq. Chrom. Rel. Technol., 24: 153-159.