The Determination of the Pyruvic Acid Content of Garlic Tissue Homogenates

Introduction

The principal compounds of intact garlic tissue which serve as precursors of the typical flavour and odour compounds are the alkyl- and alkenylcysteine sulphoxides[1,2]. The major compound found in garlic is (+)-S-allyl-L-cysteine sulphoxide (allylcysteine sulphoxide or alliin) with smaller amounts of (+)-S-methyl-L-cysteine sulphoxide (methylcysteine sulphoxide) and (+)-S-trans-(1-propenyl)-L-cysteine sulphoxide (trans-1-propenylcysteine sulphoxide or isoalliin)[3,4]. When garlic tissue is disrupted the vacuolar enzyme alliinase or alliin lyase (EC 4.4.1.4), rapidly lyases the cytosolic alk(en)yl cysteine sulphoxides[2,5] to form sulphenic acids (R-SOH)[6] ; these immediately condense to form the alkyl alkanethiosulphinates (R_1-SS(O)-R_2), the principal flavour compounds of garlic.
The formation of thiosulphinates in disrupted garlic tissue is extremely rapid with most workers agreeing that all reactions are complete within 10 minutes[7,8,9,10]. All possible combinations of 2-propene-, 1-propene-, and methanesulphenic acids result in thiosulphinates and these rearrangements are accompanied by the production of pyruvic acid and ammonia.

The large amounts of endogenous ammonia in garlic tissue render its determination unattractive, however the measurement of pyruvic acid as an indicator of pungency and flavour in both onions[9,11] and garlic[12,13] has been employed by a number of workers.
The experimental method employed here is a modification of the technique described by Schwimmer et al in which filtered onion juice is reacted with 2,4-dinitrophenylhydrazine and where the resulting dinitrophenylhydrazones are measured colorimetrically. Since this procedure actually measures total carbonyl content it was necessary to compare results obtained by this method with those of a method specific for pyruvic acid. In this latter method[14], reduced diphosphopyridine nucleotide is oxidised by pyruvic acid in the presence of excess lactic dehydrogenase and the decrease of reduced nucleotide is used as measure of pyruvic acid. Since the two methods are found to yield approximately the same values it can be concluded that the increase in
2,4-dinitrophenylhydrazone, as measured colorimetrically, can be largely attributed to the production of pyruvic acid.

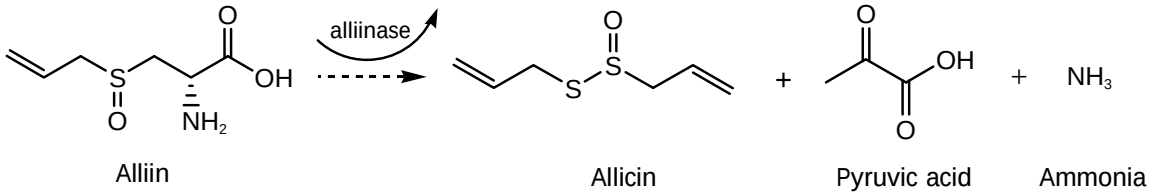

Figure 1. The formation of pyruvic acid in the alliin → allicin reaction.

Materials and Methods

***Preparation of garlic homogenates*:** Randomly chosen cloves from a number of garlic bulbs were selected, peeled and coarsely chopped with a knife. Each 100 g sample of chopped garlic was mixed by hand and 5 g samples removed for assessment.
Each 5 g sample of chopped garlic was homogenised for 2 min with 20 ml distilled water in a food blender and then made up to 1 l with distilled water. The sample was allowed to stand at room temperature for 15 min before being filtered through Whatman No. 4 filter paper.

***Controls*:** Garlic tissue contains a low level of pyruvic acid that is not a product of the alliin → allicin reaction. In order to account for this, control samples were prepared in which the enzyme alliinase had been heat deactivated. Unpeeled garlic samples, each weighing approximately 10 g, were heated in a microwave oven for 30 sec (Matsui model 170TC, λ = 2450 MHz, O/P = 650W) before being processed in the normal way.

***Determination of pyruvic acid*:** A 0.0125% 2,4-dinitrophenylhydrazine (DNPH) solution was prepared by dissolving 0.1625 g of wet DNPH powder (~30% water) in 1000 ml 2N HCl.
A 2 ml sample of the diluted, filtered homogenate was added to 1 ml of a 0.0125% solution of 2,4-dinitrophenylhydrazine in 2N HCl. After 15 min in a water bath at 37°C, 5 ml of 0.6N NaOH was added and the absorbance measured immediately on a Shimadzu model UV-160A spectrophotometer (420 nm filter, set at zero absorbance with reagent blank). The difference between the pyruvic acid content of the homogenates from unheated (P_T) and heated garlic samples (P_C) is defined as μmoles of enzymatically produced pyruvic acid (P_E) per gram garlic.

***Calibration*:** The method was calibrated using sodium pyruvate as standard.
Pyruvic acid standards were prepared using sodium pyruvate. A 10μM/ml solution was prepared by dissolving 1.1g sodium pyruvate (m.w. = 110.0g) in 1000 ml of distilled water. A subsequent ten-fold dilution gave a 1μM/ml standard solution which was further diluted to prepare calibration standards.

***Dry weight determination*:** Garlic cloves were peeled, sliced laterally into approximately 1 mm slices and then dried in an oven at 105°C for 10 h (Appendix 1).

Results

The results obtained using sodium pyruvate as standard are shown in Table 1.

Standard Solution (c) μM/ml	Absorbance 1 A_1	Absorbance 2 A_2	Mean Absorbance A_M
0.00	0.000	0.000	0.000
0.05	0.118	0.120	0.119
0.10	0.212	0.212	0.212
0.15	0.337	0.344	0.340
0.20	0.400	0.410	0.405
0.25	0.531	0.495	0.513
0.30	0.552	0.552	0.552
0.35	0.662	0.595	0.628
0.40	0.680	0.661	0.670
0.50	0.762	0.729	0.745

Table 1. Absorbance values for sodium pyruvate standard.

The relationship between molar concentration and light absorption is governed by the Beer-Lambert law[15]. This can be conveniently expressed as,

$$\log_{10} (I_o / I) = \varepsilon\, c\, l \qquad \text{or} \qquad \varepsilon = A / c\, l$$

where,

$\log_{10} (I_o / I)$ is the absorbance of the solution, (A_M)
c is the concentration of the solute (mol dm^{-3})
l is the path length of the sample (cm)
ε is the molar absorptivity (10^{-2} m^2 mol^{-1})

Since path length (l) and molar absorptivity (ε) are constants, the expression predicts a linear relationship between absorbance (A_M) and concentration (c) (Figure 2).

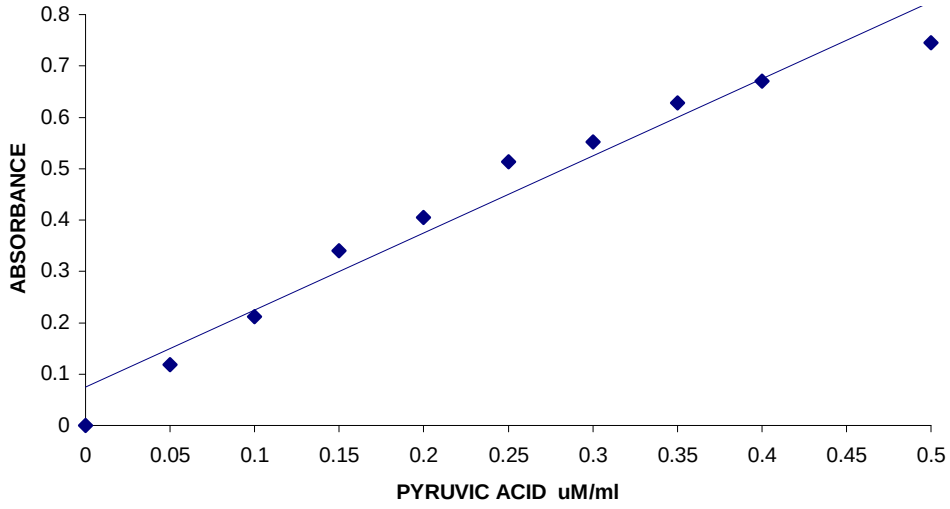

Figure 2. The linear relationship predicted by Beer-Lambert's law

In order to asses the extent of the linear relationship between the known concentrations of sodium pyruvate (c) and the corresponding absorbance values (A_W), the Pearson product moment correlation coefficient (r) was calculated[16].
The r value of the regression line is given by the following formula:

$$r = \frac{n(\sum xy) - (\sum x) - (\sum y)\ .}{\sqrt{[n\sum x^2 - (\sum x)^2][n\sum y^2 - (\sum y)^2]}}$$

$$\equiv \frac{\sum xy - nxy}{n\sigma_x\sigma_y}$$

$$= \frac{1.3133 - 1.0692}{0.2490} = 0.980$$

A value of r = 0.980 for the two sets of data indicates a high positive correlation.
If the correlation coefficient is calculated for successive data sets then it will be seen that the values decline consistently as concentration increases and suggests that the loss of linearity is a result of increasing solute-solvent interactions that are not accounted for by Beer-Lambert's law rather than practical errors such as inaccurate preparation of standard solutions or temperature effects.
Subsequent trendline analysis[17] shows that a polynomial curve fits the data accurately. The function of the calibration curve shown in Figure 4 is a polynomial of the form,

$$y = b+c_1x+c_2x^2+c_3x^3+c_4x^4\ldots\ldots\ldots c_8x^8$$

which when calculated and applied to the data in Table 1 results in perfect positive correlation (r = 1.0).

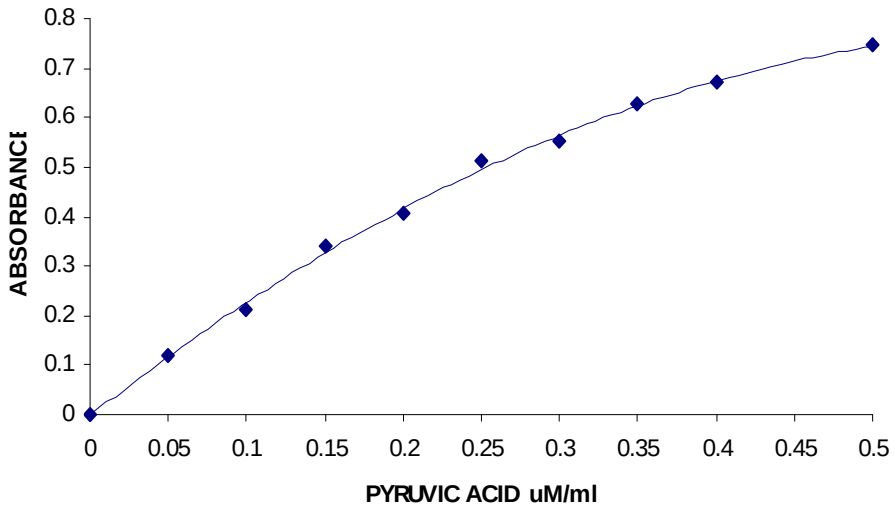

Figure 3. Calibration curve of pyruvic acid

In order to accurately determine the concentrations of pyruvic acid from absorbance measurements of unknown samples (P_C and P_T) the function y = f(x) of the calibration curve was calculated as a 9th order polynomial and applied. The results are shown in Tables 2 and 3. Because of the progressive loss of linearity above 0.3 μM/ml the use of the calibration curve has been limited to values between 0 - 0.4 μM/ml. This is a workable range for the measurements recorded during this experiment.

Garlic Clone	Absorbance Values					Pyruvic Acid
	1	2	3	4	Mean	P_C (μM/ml)
California Late	0.026	0.026	0.026	0.021	0.025	0.0109
Carpathian	0.040	0.042	0.044	0.046	0.043	0.0187
Cristo	0.058	0.068	0.048	0.047	0.055	0.0239
Dominics	0.032	0.026	0.026	0.027	0.028	0.0122
French Red	0.062	0.066	0.059	0.041	0.057	0.0248
Freudenberger	0.046	0.041	0.044	0.029	0.040	0.0174
Georgia Fire	0.064	0.065	0.052	0.053	0.059	0.0257
German Red	0.042	0.042	0.045	0.044	0.043	0.0187
Inchelium	0.052	0.076	0.038	0.052	0.055	0.0239
Israeli	0.032	0.032	0.024	0.026	0.029	0.0126
Leningrad	0.039	0.030	0.032	0.028	0.032	0.0139
Marino	0.037	0.036	0.039	0.037	0.037	0.0161
Morado	0.026	0.029	0.030	0.026	0.028	0.0122
Music	0.039	0.042	0.036	0.035	0.038	0.0165
Red Janice	0.047	0.039	0.043	0.027	0.039	0.0170
Red Rezan	0.054	0.059	0.050	0.046	0.052	0.0226
Roja	0.048	0.044	0.043	0.036	0.043	0.0187
Russian Redstreak	0.031	0.031	0.030	0.029	0.030	0.0130
Weingarten	0.060	0.060	0.056	0.055	0.058	0.0253
Yerina	0.044	0.036	0.036	0.049	0.041	0.0178
Yugoslavian	0.064	0.066	0.040	0.039	0.052	0.0226

Table 2. Absorbance values for control garlic homogenates

Garlic Clone	Absorbance Values					Pyruvic Acid
	1	2	3	4	Mean	P_C (µM/ml)
California Late	0.622	0.628	0.632	0.632	0.629	0.3505
Carpathian	0.441	0.447	0.454	0.453	0.449	0.1992
Cristo	0.661	0.671	0.671	0.667	0.668	0.3959
Dominics	0.542	0.554	0.555	0.557	0.552	0.3000
French Red	0.524	0.531	0.542	0.542	0.535	0.2789
Freudenberger	0.574	0.582	0.583	0.583	0.581	0.3271
Georgia Fire	0.633	0.648	0.675	0.678	0.659	0.3800
German Red	0.606	0.617	0.626	0.626	0.619	0.3457
Inchelium	0.403	0.409	0.413	0.413	0.410	0.1997
Israeli	0.533	0.542	0.543	0.551	0.542	0.2879
Leningrad	0.620	0.629	0.624	0.624	0.624	0.3480
Marino	0.519	0.525	0.529	0.529	0.526	0.2670
Morado	0.644	0.656	0.642	0.647	0.647	0.3644
Music	0.618	0.625	0.606	0.601	0.613	0.3432
Red Janice	0.415	0.422	0.419	0.422	0.420	0.1989
Red Rezan	0.586	0.599	0.586	0.592	0.591	0.3333
Roja	0.626	0.645	0.655	0.654	0.645	0.3624
Russian Redstreak	0.536	0.546	0.546	0.541	0.542	0.2879
Weingarten	0.626	0.646	0.646	0.647	0.641	0.3588
Yerina	0.589	0.603	0.610	0.611	0.603	0.3391
Yugoslavian	0.600	0.610	0.609	0.607	0.607	0.3408

Table 3. Absorbance values for test garlic homogenates.

Garlic Clone	Pyruvic Acid Concentration (µM/ml)			Dry Wt[a] (%)	Pyruvic Acid Conc (µM/g)[b]		Thiosulphinate (µM/g fresh wt)[c]	
	P_C	P_T	P_D		Fresh Wt	Dry Wt	Fresh Wt	Dry Wt
California Late	0.0109	0.3505	0.3396	35.52	67.92	191.22	33.96	95.61
Carpathian	0.0187	0.1992	0.1805	36.29	36.10	99.48	18.05	49.74
Cristo	0.0239	0.3959	0.3720	36.45	74.40	204.12	37.20	102.06
Dominics	0.0122	0.3000	0.2878	38.46	57.56	149.66	28.78	74.83
French Red	0.0248	0.2789	0.2541	35.81	50.82	141.92	25.41	70.96
Freudenberger	0.0174	0.3271	0.3097	38.81	61.94	159.60	30.97	79.80
Georgia Fire	0.0257	0.3800	0.3543	36.28	70.86	195.31	35.43	97.66
German Red	0.0187	0.3457	0.3270	37.00	65.40	176.76	32.70	88.38
Inchelium	0.0239	0.1997	0.1758	35.77	35.16	98.29	17.58	49.15
Israeli	0.0126	0.2879	0.2753	38.26	55.06	143.91	27.53	71.96
Leningrad	0.0139	0.3480	0.3341	39.37	66.82	169.72	33.41	84.86
Marino	0.0161	0.2670	0.2509	37.32	50.18	134.46	25.09	67.23
Morado	0.0122	0.3644	0.3522	35.45	70.44	198.70	35.22	99.35
Music	0.0165	0.3432	0.3267	37.97	65.34	172.08	32.67	86.04
Red Janice	0.0170	0.1989	0.1819	35.04	36.38	103.82	18.19	51.91
Red Rezan	0.0226	0.3333	0.3107	36.97	62.14	168.08	31.07	84.04
Roja	0.0187	0.3624	0.3437	35.51	68.74	193.58	34.37	96.79
Russian Redstreak	0.0130	0.2879	0.2749	34.69	54.98	158.49	27.49	79.24
Weingarten	0.0253	0.3588	0.3335	39.16	66.70	170.33	33.35	85.16
Yerina	0.0178	0.3391	0.3213	34.44	64.26	186.59	32.13	93.29
Yugoslavian	0.0226	0.3408	0.3182	36.54	63.64	174.17	31.82	87.08

Table 4. Pyruvic acid and thiosulphinate values for garlic homogenates

[a] *Refer Appendix 1.* [b] *Pyruvic acid concentration = P_D x 200 (see Materials & Methods).*

[c] Calculation based on ½ mole thiosulphinate for each mole of pyruvic acid produced.

Discussion

The method described has provided consistent data as shown by the data analysis in Table 5. The coefficients of variation for all of the absorbance measurements ranged from 0.6% - 3.3% with a mean value of 1.28%.

The colour reaction upon which this test depends is time and temperature sensitive and consistency in these parameters should be strict throughout. Once fixed the colour appears to be stable for approximately 10 minutes at room temperature indicating that colorimetric readings should be taken immediately after the sample is removed from the water bath.

The preparation of control samples has been described by a number of workers[9,11] and although the method used here is a convenient approach, account should be taken of the moisture loss associated with the use of the microwave oven (~20%).

Clearly the solute-solvent interaction that causes the loss of linearity in the absorbance/concentration data is a significant problem and one which could result in conversion errors. Some of the higher absorbance readings obtained in this experiment were approaching the upper usable limit of the calibration curve and sample concentrations should in future be adjusted so that they are accommodated by the linear section of the graph.

Although this method of analysis is primarily developed to measure the pungency of onions, its value in providing a comparative measure for garlic clones is evident. The major limitation of using pyruvic acid determination is its lack of specificity, i.e. it is an indirect measure of total

Garlic Clone	Absorbance Values (au)		
	Mean	Standard Deviation ($x10^{-3}$)	Coefficient of Variation (%)
California Late	0.629	4.73	0.75
Carpathian	0.449	6.02	1.34
Cristo	0.668	4.73	0.71
Dominics	0.552	6.78	1.23
French Red	0.535	8.84	1.65
Freudenberger	0.581	4.36	0.75
Georgia Fire	0.659	21.70	3.29
German Red	0.619	9.50	1.53
Inchelium	0.410	4.73	1.15
Israeli	0.542	7.36	1.36
Leningrad	0.624	3.69	0.59
Marino	0.526	4.73	0.90
Morado	0.647	6.19	0.96
Music	0.613	10.97	1.79
Red Janice	0.420	3.32	0.79
Red Rezan	0.591	6.19	1.05
Roja	0.645	13.44	2.08
Russian Redstreak	0.542	4.79	0.88
Weingarten	0.641	10.18	1.59
Yerina	0.603	10.15	1.68
Yugoslavian	0.607	4.51	0.74

Table 5

thiosulphinate and cannot provide any differentiation of the individual alk(en)yl thiosulphinates present in disrupted garlic tissue. Although this method lacks the specificity of HPLC it has been

developed further by a number of workers[12,13,18] and while the modified methods provide greater linearity and stability they are somewhat cumbersome and time-consuming. The method described by Jager[12] measures the DNPH of pyruvic acid after differential extraction to remove unreacted hydrazine derivative and non-pyruvate hydrazones.

Determination	Literature	Method	Concentration (μM/g fresh weight)	
			Pyruvic Acid	Thiosulphinate
Pyruvic acid	Present	DNPH derivative of pyruvate	35 - 60	(17 - 30)
Pyruvic acid	(12, 13)	DNPH derivative of pyruvate	47 - 63	
Thiosulphinates	(17)	Si - HPLC	-	14 - 36
Allyl mercaptan	(18)	GC, headspace.	-	15 - 30
Diallyl sulphide	(19)	GC	-	12 - 22
Allicin	(20)	C18 - HPLC	-	22 - 30
Allicin	(21)	C18 - HPLC	-	18 - 37
Thiosulphinates	(22)	Si - HPLC	-	26 - 40

Table 6. Comparison of results of pyruvate / thiosulphinate content of garlic clove homogenates.

Calibration is afforded by the use of highly purified, recrystallised DNPH of pyruvic acid dissolved in ammonia. Similarly, Kornberg[18] describes a method based on the oxidation of reduced diphosphopyridine nucleotide (DPNH) by pyruvate in the presence of excess lactic dehydrogenase although a stoichiometric relation between standard pyruvate added and DPNH oxidised was obtained only when freshly prepared solutions of DPNH were used. Adaptation of the current method has avoided the complexities associated with these other methods and has, within known limits, provided consistent data.

Over the past fifty years workers have adopted a number of diverse methods in attempts to quantify the pyruvate and thiosulphinate levels in various *Allium* species. A summary of the methods used and the quantities of pyruvate/thiosulphinate detected are shown in Table 6.
The original method of Jäger[12] is in excellent agreement with current C18- and Si-HPLC results although the high value reported by Miething[19] is possibly due to decomposition of the allicin standard in ether.[20] The results of this study are in good general agreement with other workers irrespective of the methodology employed.

Statistical Analysis

Having determined the thiosulphinate (pyruvate) values for each of the samples clones, the results were filtered according to isozyme group and the data analysed to see if the thiosulphinate values of isozyme groups were significantly different. An analysis of variance test (ANOVA) was applied to the data as shown in Tables 7 and 8.

Garlic Clone	Isozyme Group		
	1	**3**	**4**
California Late			33.96
Carpathian	18.05		
Cristo			37.20
Dominics	28.78		
French Red	25.41		
Freudenberger			30.97
Georgia Fire		35.43	
German Red	32.70		
Inchelium			17.58
Israeli	27.53		
Leningrad		33.41	
Morado			35.22
Music		32.67	
Red Janice			18.19
Red Rezan		31.07	
Roja	34.37		
Russian Redstreak			27.49
Yerina	32.13		
Yugoslavian	31.82		
Count	8	4	7
Sum	230.8	132.6	200.6
Mean	28.85	33.15	28.66
Variance	27.87	3.27	63.86

Table 7

ANOVA

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F	P-value
Between groups	60.8	2	30.43	0.828	0.455
Within Groups	588.0	16	36.75		
Total	648.9	18			

Table 8

The analysis indicates that isozyme group is unlikely to have any effect on total thiosulphinate value when clones from those groups are grown under similar environmental and cultural conditions to those used in this study.

REFERENCES

[1] Stoll, A., Seebeck, E. (1947). *Experientia*, **3**, 114-115.
[2] Stoll, A., Seebeck, E. (1949). *Helv. Chim. Acta*, **32**, 197-205.
[3] Fujiwara, M., Yoshimura, M., Tsuno, S., Murakami, F. (1958). *J. Biochem*., **45**, 141-149.
[4] Lawson, L. D., Hughes, B. G. (1990). *Planta Med*., **56**, 589.
[5] Lancaster, J. E., Colin, H. A. (1981). *Plant Sci. Lett*., **22**, 169-176.
[6] Block, E., O'Connor, J. (1974). *J. Am. Chem. Soc*., **96**, 3929-3944.
[7] Lawson, L. D. (1993). *Am. Chem. Soc*., **21**, 306-330.
[8] Lawson, L. D., Wood, S. G., Hughes, B. G. (1990). *Planta Med*., **57**, 263-270.
[9] Schwimmer, S., Weston, W. J. (1961). *Agric. Fd. Chem*., **9 (4)**, 301-304.
[10] Carson, J. F. (1987). *Food Reviews International*, **3 (1/2)**, 71-103.
[11] Schwimmer, S., Guadagni,D. G. (1962). *J. Food Sci*., **27**, 94-97.
[12] Jäger, H., (1955). *Arch. Pharm*., **228**, 145-148.
[13] Alfonso, N., Lopez, H., (1959). *Contribs. Client. Inst. Mexicano Invest. Technol*., **59 (2).**
[14] Schwimmer, S., Mazelis, M. (1963). *Arch. Biochem. Biophys*., **100**, 66.
[15] Kemp, W. (1978) *Organic Spectroscopy*. Macmillan Press Ltd.
[16] Harper, W. M. (1971) *Statistics.* Macdonald & Evans Ltd.
[17] Mannall, G., Kenwood, M. (1994) *Pure Mathematics*. Heinemann.
[18] Block, E., Putman, D., Zhao, S-H. (1992). *J. Agric. Food Chem*., **40**, 2431-2438.
[19] Koch, J., Berger, L., Vieregge-Reiter, C. (1989). *Planta Med*., **55**, 327-331.
[20] Müller, B. (1989). *Dtsch. Apoth. Ztg*., 129, 2500-2504.
[21] Iberl, B., Winkler, G., Knobloch, K. (1990). *Planta Med*., **56**, 202-211.
[22] Iberl, B., Winkler, G., Müller, B., Knobloch, K. (1990). *Planta Med*., **56**, 320-326.
[23] Lawson, L. D. (1991). *Planta Med.,* **57**, 263-270.
[18] Kornberg, A. (1955) *Methods in Enzymology*, **1**, 444.
[19] Miething, H. (1985) *Dtsch. Apoth. Ztg*., **125**, 2049-2050.
[20] Voigt, M., Wolf, E. (1986) *Dtsch. Apoth. Ztg.,* **126**, 591-593.