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REVIEW ARTICLE

ORIGINAL PAPERS

INFLUENCE OF SELECTED PARAMETERS ON THE ISOELECTRIC ADSORPTIVE BUBBLE SEPARATION (IABS) OF POTATO PROTEINS

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SUMMARY

The effect of pH on the total protein content and the content of special protein fractions during adsorptive bubble separation was determined. Potato (*Solanum tuberosum var. agria*) juice was chosen as a model system because of its foaming capacity and its relevancy as wastewater in the starch industry. Two operation modes of the adsorptive bubble chromatography method - batch and continuous mode - were compared with respect to the potential for protein enrichment. All samples were analysed for their total protein content, and two samples in the acidic and alkaline range also via 2D-electrophoresis with respect to their protein composition. It could clearly be demonstrated that special protein fractions were nearly totally enriched into the foam at pH 3 and 9 (i.e. values of pI 3 and pI 9), while only traces of them remained in the residual potato juice. During continuous I-ABS, protein enrichment ratios of up to R=3.36 could be obtained compared to the batch method.

KEYWORDS: Isoelectric adsorptive bubble separation (I-ABS); foam fractionation; protein separation; *Solanum tuberosum var. agria*; 2D-electrophoresis.

INTRODUCTION

Adsorptive bubble separation (ABS), though still rarely used, is a suitable method for the enrichment of surface-active solutes, such as enzymes and other proteins, under mild conditions (Ostwald and Siehr, 1937; Karger et al., 1967; Lemlich, 1972; Maas, 1974; Uraizee and Narsimhan, 1990; Narsimhan and Uraizee, 1995). Because of its additional (low mechanical stress and low maintenance costs as well as the avoidance of the use of hazardous organic solvents and additives) this method may be an alternative to the more generally used techniques, such as ultrafiltration, gelfiltration, ion exchange, precipitation and coagulation, especially, on an industrial scale.

Gas bubbles, produced by a stream of an inert gas via a porous glass frit dipped into the liquid pool at the bottom of a glass column, form a stable foam above the solution (Figure 1). The foam rises up the column and the liquid part of the foam drains due to gravity. The separation takes place by means of selective adsorption at the liquid-gas interface of ascending bubbles (Cannon and Lemlich, 1972). The adsorption at the interface effects a surface denaturation with partial unfolding of the protein molecules uncovering additional amino acid side chains. The interaction of these partly unfolded protein molecules, which can be improved by a slight net charge of the proteins, adds to the stabilization of the film (Belitz and Grosch, 1992). The redissolved foam forms a solution with enriched surface-active proteins. In most cases, these are denatured and biologically inactive, but may be used for technical purposes or nutrition.

The foaming capacity depends on the molecular properties, mainly the hydrophobicity of the (Townsend and Nakai, 1983). With rising hydrophobicity the foaming capacity increases. This can be influenced by the pH value. If the net charge is zero, i.e. the isoelectric point (pI) of this protein is reached and the molecule is at its maximum of hydrophobia (Thomas and Willer, 1977; Liu et al., 1995). Different operation modes have been developed for the ABS; Pinfold (1970) has given an overview of all ABS methods.

The main advantages of this technique are low energy, investment, and operational costs, a simple apparatus, high selectivity and efficiency, especially, in diluted solutions. Although the technique is known since 1937 (Ostwald and Siehr, 1937), there is still a lack of investigations with respect to the influence of the pH value on protein enrichment. Also, there are no investigations on a pH-dependent enrichment of special protein fractions - the so-called "Isoelectric Adsorptive Bubble Chromatography (I-ABC)".

Not only one protein (Ahmad, 1975; Britten and Lavoie, 1992; Liu et al., 1995), but also certain protein fractions from complex mixtures can be enriched in foam.

The world population is provided with millions of tons of high-quality potato proteins per annum. Combined with other foodstuffs and acceptably flavoured, potato proteins may play an important role in the human nutrition. The amino acid composition is well balanced and the quality of the proteins is comparable with those of the egg (Knorr, 1977 and 1978).

During industrial processing of potato tubers, e.g. in the starch industry, high amounts of potato juice with a residual content of high-quality proteins in the range of 0.1-0.2% are produced.

Hitherto, these proteins were either used for animal feeding or discharged, resulting in a high wastewater burden (Brown et al., 1990). A cost-efficient method for the enrichment or isolation of protein fractions is of high economical and ecological interest. These recovered proteins could be utilised in the production of protein enriched food, especially in the field of "senior nutrition".

A further application area of the enriched protein fractions might be the food and fertilizer industry. Some parts could be used as basic material for glues. The removal of the proteins from potato wastewater would provide the additional advantage of diminishing the DOC load and the discharge of these wastewaters and the intensification in environmental protection in the food industry.

MATERIALS AND METHODS

Materials

The protein assay kit was obtained from Sigma (Deisenhofen, Germany). Multiphor II horizontal electrophoresis apparatus, Dalt-multiple vertical electrophoresis apparatus, EPS 3500 XL power supply, Multitemp II thermostatic circulator, Immobiline II chemicals, Pharmalyte (pH range 3-10), IPG buffers, Immobiline DryStrip-Kit, acrylamide, bisacrylamide, ammonium persulfate, TEMED, CHAPS, and urea were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Acrylamido buffers p*K* 1.0, p*K* 10.3 and p*K* > 13 were a gift from Bengt Bjellqvist (Amersham Pharmacia Biotech). Silicone oil was received from Serva (Heidelberg, Germany). All other chemicals (analytical grade) for electrophoresis and for silver staining were obtained from Merck (Darmstadt, Germany). The water was double-distilled.

Potato tubers (*Solanum tuberosum var. agria*) were purchased on the local market (Grassl, Mintraching-Grüneck, Germany).

Preparation of potato juice

50 kg of potato tubers (*Solanum tuberosum var. agria*) were washed and pressed out with a "Speidel-Hydro-Presse-90 l" (Ofterdingen, Germany). The potato juice was filtered and portions of about 20 ml were stored at -50°C until analysis.

Protein enrichment by I-ABS

Potato (*Solanum tuberosum var. agria*) juice portions were defrosted and diluted 1:20 with water. In these stock solutions for foam fractionation the pH values were adjusted between 3 and 11. During batch I-ABS (Figure 1) of the pH-different potato juice solutions, samples of foam overflow were taken every 15 min and total protein content of the initial liquid and the foam samples was investigated. The glass column (height 94 cm, i. d. 4 cm) was filled with a portion (about 450 ml) of the diluted potato juice. Then nitrogen gas was sparged into the liquid pool (500 ml min^{-1}). The foamate was collected in a vessel. 1 ml aliquots of collapsed foam were used in sample preparation, either for protein determination or for 2 D-electrophoresis.

During continuous I-ABS (Figure 2) of the pH-different stock solutions, samples of foam were taken every 10 min and analysed similarly as described above. The storage jar and the glass column were filled with diluted potato juice. Additionally, potato juice was fed back continuously into the foam and, simultaneously, a part of the bulk liquid was removed.

The bulb (diameter 12 cm) on the upper part of the column intensified the drainage effects taking place during the rise of the foam and enhanced protein enrichment by about 10-15%. After foam breaking, the collected solution (foamate) was split into two parts. One part of the foamate - the reflux - was led back into the foam column; the other part was used for protein determination. The operating conditions of continuous I-ABS were: gas flow 500 ml N_2 min^{-1}; product feed 17 ml min^{-1}; removal rate 4 ml min^{-1} and reflux 7 ml min^{-1}.

Determination of total protein content

Protein determination was performed with a protein assay kit (Sigma) spectrophotometrically (Gschwendtner, 2000). An alkaline cupric tartrate reagent forms a purple-colored complex after addition of phenol reagent. Absorbance was measured at 540 nm. The protein concentration was determined by means of a calibration curve. To eliminate the influence of varying concentrations in the initial liquid protein pool, the protein enrichment ratio R was calculated, which is defined as

$$R = \frac{c_F}{c_A}$$

where c_F is the protein concentration of the foam sample and c_A is the protein concentration of the initial liquid.

The total protein content of the initial potato juice stock solution was in the range of 500 - 640 µg ml^{-1} (see Table 1). The differences were due to varying percentages of irreversibly denaturated proteins after pH adjustment. These proteins were filtered off during preparation of the initial liquid for I-ABS.

Gelelectrophoretic determination of protein fractions

Gels: IPG gels 3-12 as well as vertical gels were cast as described previously (Görg et al., 1988; 1995; Görg and Weiss, 1998; Görg, 1991; 1998).

Performance of 2D-electrophoresis*:*

0.91 g urea, 40 mg CHAPS, 40 µl Pharmalyte 3-10, and 1 mg Pefablock SC were dissolved in 1 ml samples. After shaking for 5 min, 20 mg Dithiothreitol (DTT) was added. 100 µl aliquots of the sample solutions were used for IPG-IEF. 2D-electrophoresis was performed as described previously (Görg et al., 1999).

RESULTS

Protein enrichment during batch I-ABS

During all the experiments a more or less enrichment of proteins in the foam could be observed (Table 1). As expected, the lowest enrichment values were obtained after 10 min of batchwise mode. Varying pH values in this case have influenced the enriching operation and a maximum value of 960 µg ml^{-1} of total proteins in the collapsed foam was achieved at pH 9 after 45 min of foaming. Additionally, the experiments showed that total proteins accumulate nearly identically and not to a sufficient degree, comparing all the enrichment ratios (R) at the different pH values and foaming times used. The average values of R in the acidic and alkaline range, calculated on basis of the corresponding single R values in Table 1, were 1.54 (pH 3-6) or 1.55 (pH 8-11) after 45 min of foaming and 1.57 (pH 3-6) or 1.48 (pH 8-11) after 60 min. These values also clearly demonstrate that protein concentrations in the foamate increased time-dependently.

After 15, 30, 45, and 60 min of foaming, concentrations of 560-780, 680-872, 807-960, and 832-880 µg ml^{-1}, respectively , have been determined,. The optimal time to effect highest protein enrichment was 45 min. Prolonging the foaming period up to 60 min at different pH values did not remarkably influence the yield of total proteins, but, in fact, slightly decreased their foamate concentration in the alkaline range (pH 8-11), caused by a gradual occurrence of a too low protein content in the initial pool.

In addition, the protein compositions of I-ABS´s at pH 3 and 9 were examined by 2D-electrophoresis. The results concisely emphasized that certain potato protein fractions could be selectively enriched around pI 3 and 9 in correlation to the adjusted pH values of 3 and 9, respectively.

The comparison of the IPG Dalts of the initial liquid pool with the corresponding foam-samples ascertained that certain protein fractions around pI 3 (Figure 3) have been, in correlation with the adjusted pH value 3, almost completely enriched into the foam. This was confirmed by a total lack of these protein fractions in the residual liquid (Figure 3). With regard to the molecular weight, it was shown that proteins up to 30 kDa were preferably enriched into the foam.

A similar result, as described above, is shown in Figure 4, where the initial potato juice was adjusted to pH 9 and several protein fractions with a pI around 9 have been almost completely enriched into the foam. This result was also affirmed by 2D-electrophoresis of the remaining liquid. It was selectively deproteinized and, again, only these protein fractions up to 30 kDa were absent, because they have tended to accumulate in the foam.

Protein enrichment during continuous I-ABS

Protein enrichment in collapsed foam has been clearly demonstrated by all experiments but with the exception of all 10 min foaming periods, which evidenced low enrichment ratios from 0.90 to 1.40 (Table 2). The enriching effect was strongly pH-dependent as shown by a maximum enrichment (2350 µg ml^{-1}, R = 3.36) at pH 9 and a minimum one (1300 µg ml^{-1}, R = 1.35) at pH 6 after 40 min of foaming (Figure 5, Table 2).

Furthermore, enrichment effectiveness was highest in the alkaline pH range (>7 - 11) after 20 to 40 min of foaming, most evidently at pH 9 (1480 to 2350 µg ml^{-1}).

It could be unambiguously deduced, as already observed during batchwise I-ABS, that protein concentrations in the foamate were more or less increased with increasing operation time. At pH 3, already after 20 min of continuous foam fractionation a steady-state seems to be achieved, because enrichment rate is nearly negligible. After foaming periods of 10, 20, 30, and 40 min the protein concentrations in the foamate varied between 648 - 1180, 1148 - 1740, 1350 - 2280, and 1300 - 2350 µg ml^{-1}, respectively.

Comparison of batch vs. continuous I-ABS

It has been shown experimentally that higher protein enrichment was achieved with the continuous mode, when varying only the pH values and operation time, but not the other influencing parameters. The highest available concentration of total proteins in foamate under continuous operational conditions was 2350 µg ml^{-1} compared to 960 µg ml^{-1} with batchwise mode.

Table 1 and Figure 6 impressively prove that the enrichment rates during batch I-ABS were generally very low (concentration of initial liquid protein pool 500 - 640 µg ml^{-1}, maximum foamate concentration 960 µg ml^{-1}).

FIGURE 1 - Batch apparatus for isoelectric adsorptive bubble separation (IABS)

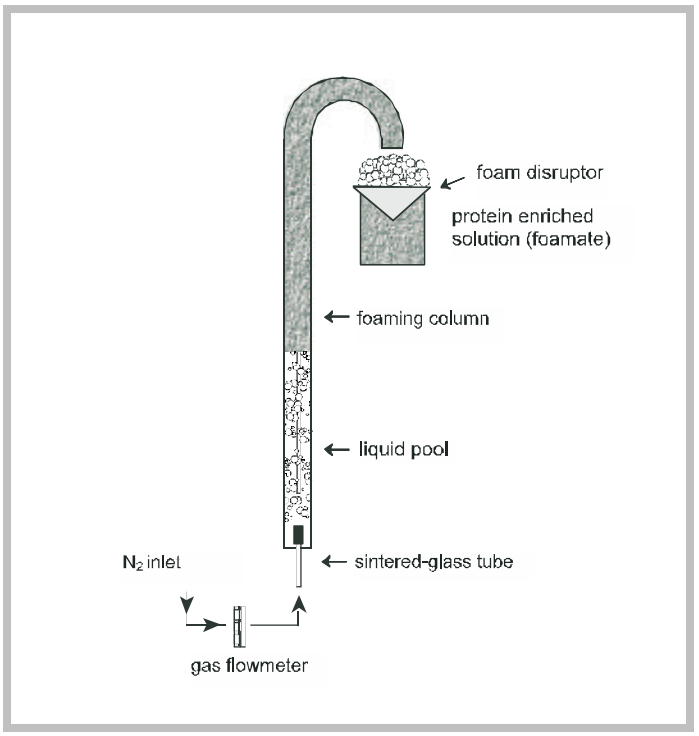


FIGURE 2 - Continous apparatus for isoelectric adsorptive bubble separation

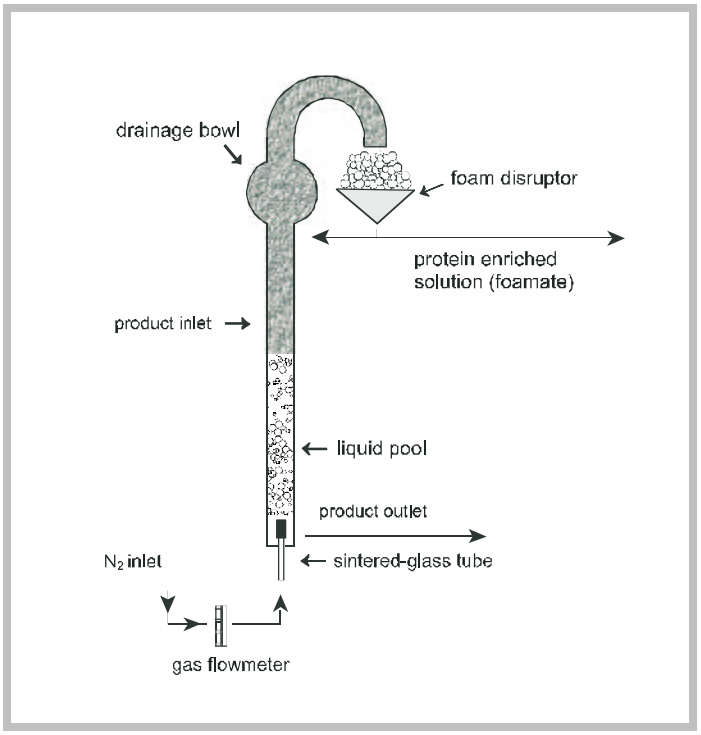


TABLE 1 - Protein concentrations and enrichment ratio R of different samples during batch I-ABS

pH	c_A [µg/ml]	t=15 min c_F [µg/ml]	R	t=30 min c_F [µg/ml]	R	t=45 min c_F [µg/ml]	R	t=60 min c_F [µg/ml]	R
3	612	696	*1.14*	780	*1.27*	807	*1.32*	832	*1.36*
4	500	576	*1.15*	736	*1.47*	880	*1.76*	880	*1.76*
5	532	560	*1.05*	732	*1.38*	856	*1.61*	888	*1.67*
6	540	632	*1.17*	680	*1.26*	800	*1.48*	832	*1.54*
7	632	636	*1.01*	760	*1.20*	848	*1.34*	832	*1.32*
8	640	736	*1.15*	868	*1.36*	912	*1.43*	880	*1.38*
9	600	780	*1.30*	872	*1.45*	960	*1.60*	880	*1.47*
10	560	736	*1.31*	868	*1.55*	920	*1.64*	868	*1.55*
11	576	704	*1.22*	868	*1.51*	880	*1.53*	868	*1.51*

c_A = concentration in initial solution; c_F = concentration in foamate

TABLE 2 - Protein concentrations and enrichment ratio R of different samples during continuous I-ABS

pH	c_A [µg/ml]	t=10 min c_F [µg/ml]	R	t=20 min c_F [µg/ml]	R	t=30 min c_F [µg/ml]	R	t=40 min c_F [µg/ml]	R
3	840	1180	*1.40*	1700	*2.02*	1620	*1.93*	1800	*2.14*
4	860	900	*1.05*	1148	*1.33*	1580	*1.84*	1440	*1.67*
5	900	1064	*1.18*	1308	*1.45*	1350	*1.50*	1440	*1.60*
6	960	1160	*1.21*	1340	*1.40*	1380	*1.44*	1300	*1.35*
7	852	852	*1.00*	1500	*1.76*	2280	*2.68*	2050	*2.41*
8	720	648	*0.90*	1520	*2.11*	2120	*2.94*	1930	*2.68*
9	700	728	*1.04*	1480	*2.11*	2000	*2.86*	2350	*3.36*
10	800	810	*1.01*	1520	*1.90*	2100	*2.63*	2050	*2.56*
11	800	860	*1.08*	1740	*2.18*	2180	*2.73*	2030	*2.54*

c_A = concentration in initial solution; c_F = concentration in foamate

FIGURE 3 - 2D-electrophoresis of initial liquid, foam samples and remaining liquid after isoelectric adsorptive bubble separation (IABS) procedure at pH 3

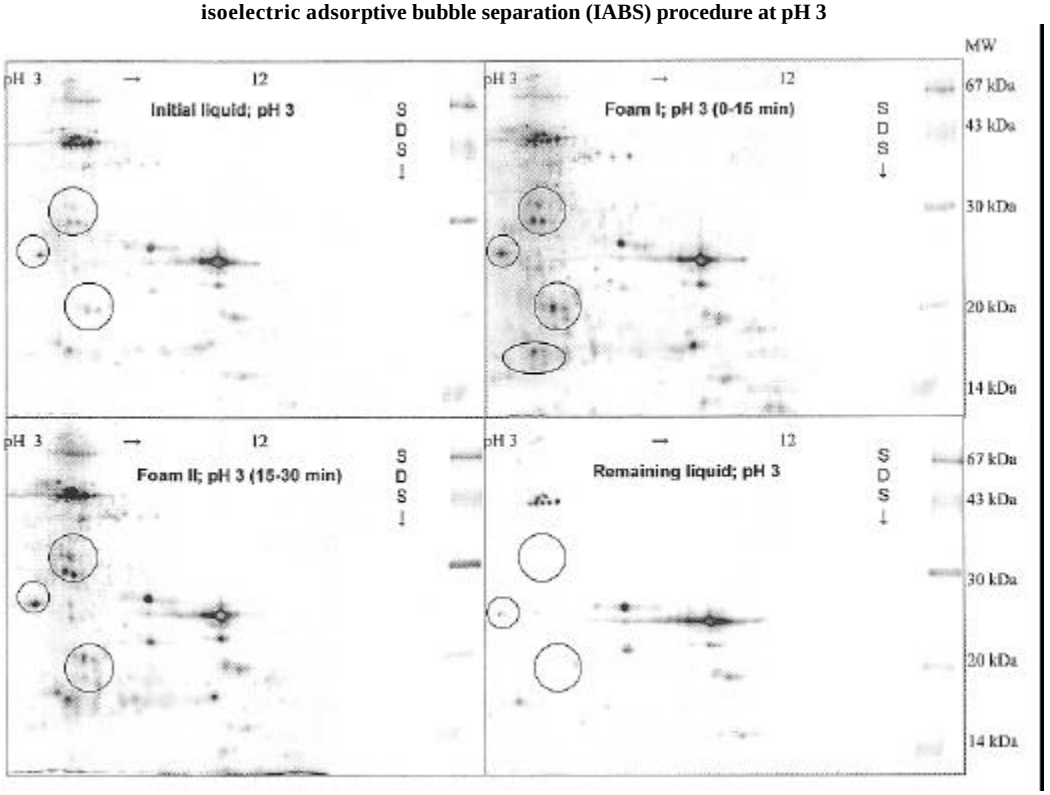


FIGURE 4 - 2D-electrophoresis of initial liquid, foam samples and remaining liquid after isoelectric adsorptive bubble separation (IABS) at pH 9

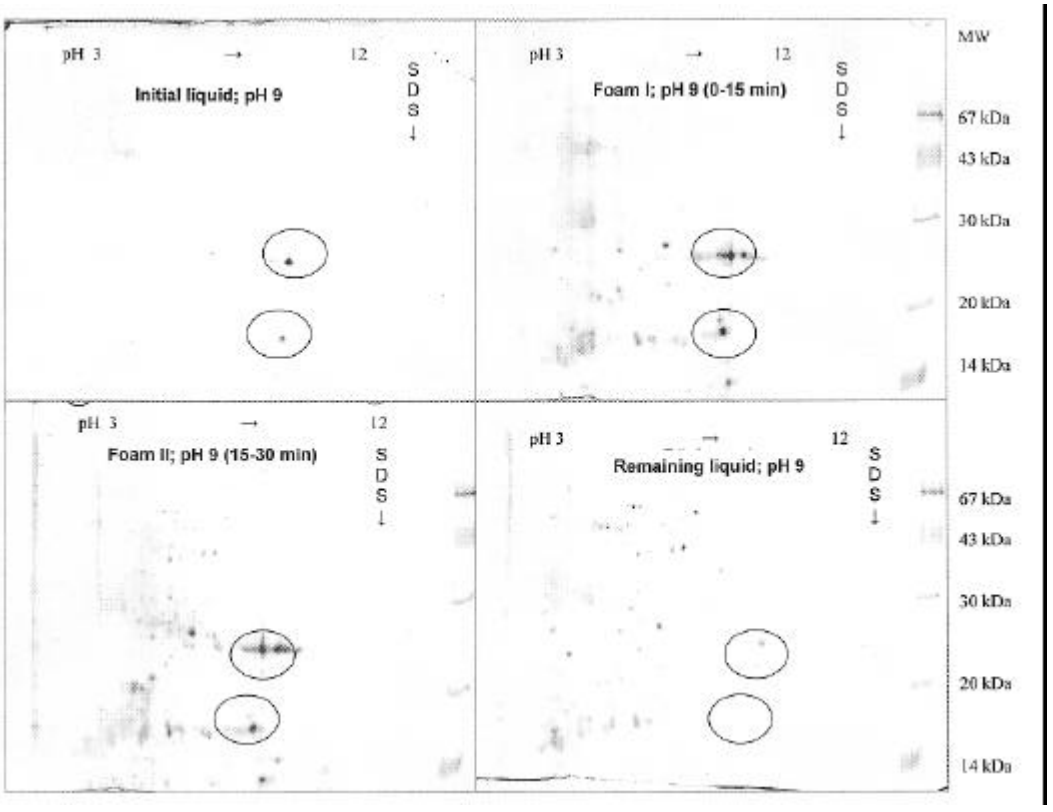


FIGURE 5 - Comparison of batch I-ABS at pH 6 with pH 9.

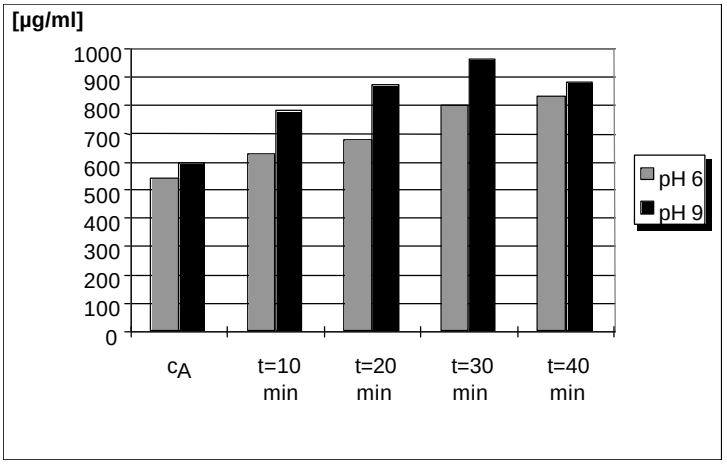


FIGURE 6 - Comparison of continuous I-ABS at pH 6 with pH 9.

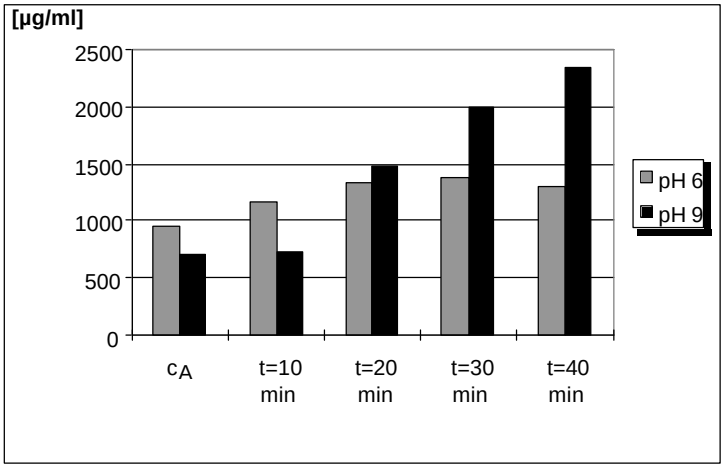
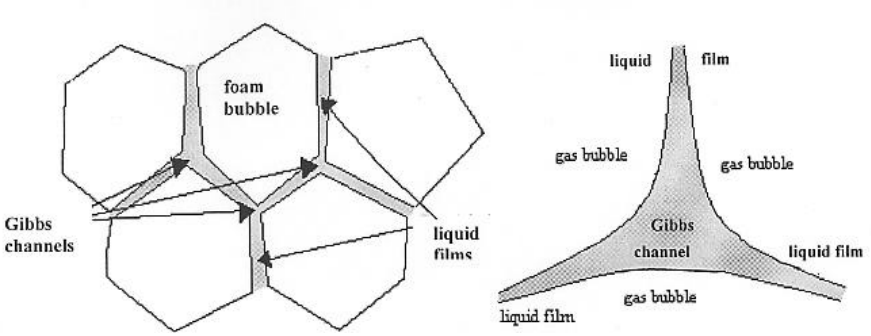


FIGURE 7 - Schematic profile of the Gibbs channel in polyhedronic foam (clustered and individual)



DISCUSSION

The above-mentioned results indicate that enriching of proteins using adsorptive bubble separation techniques, demonstrated with potato juice as a model solution, was mainly influenced by parameters, such as pH and foaming time. The maximum of enrichment was up to 3.36 times during continuous I-ABS. This mode of operation is much more effective than batch I-ABS, where an enrichment ratio R of only 1.76 was obtained.

However, experiments with our laboratory foaming plant equipped with a bulb to optimize drainage and enhance protein enrichment, a feedback of foamate to the foam column for a further enrichment and a jar to provide the liquid pool with fresh stock solution at a rate of 15 ml min^{-1} during foaming procedure, evidenced the main advantages of continuous I-ABS. Time-consuming operations such as the start-up period until equilibrium or emptying, refilling and cleaning procedures of the plant might be avoided. Furthermore, a plant capacity-dependent long-term enrichment of proteins at a constantly high concentration level could be verified. This is a well-promising basis to realize industrial scale-up under economic and ecological aspects.

In numerous preexperiments with our laboratory plant, I-ABS was optimized in order to elucidate the importance and to minimize or optimize the influence of the following important parameters on protein enrichment:

- In order to understand the foam-forming process, a short and summarized overview has to be given. Proteins are very complex and surface-active molecules possessing hydrophilic and hydrophobic sections. At first, a reason for the stabilisation of bubble surfaces is the gaining of extra energy if a nonpolar hydrophilic side chain of a protein is removed from an aqueous to a more nonpolar environment (the so-called entropy effect). The segregation of a hydrophobic side chain is an important factor in stabilizing the proteins in aqueous solution. Hence, the protein is exposed to an interface, e.g. initial stock solution/N_2 gas. This stresses the hydrophobic-hydrophilic bonding and, as a result of this stress, may break the bonds and denature the protein causing unfolding processes. The basic principle for protein enrichment in foam during I-ABS is that drainage effects take place.

  Leonard and Lemlich (1965) have discussed a realistic model, where the foam bubbles are considered to be regular dodecahedra bounded by narrow capillaries, the so-called Plateau (1861) borders or Gibbs (1931) channels of the cross sections as shown in Figure 7. Under equilibrium conditions in such a polyhedronic foam never more than three liquid films (thickness 10 nm to 1 µm), which are bordered on both sides by an interface-active thin film of proteins, come into contact creating a liquid pool in the triangular-shaped cross sections of the horizontal-oriented Gibbs channels (Figure 7, right side). These channels form a connected system filled with interstitial liquid. In describing liquid flow through a polyhedric foam, a distinction has to be made between the drainage mechanisms for the two main building blocks of such a foam: the thin liquid films and the Gibbs channels. The basic principle for protein enrichment in foam during I-ABC is that drainage effects take place. Gravity is the driving force for drainage (interstitial flow) of the thin liquid film, which is stabilized with surfactants such as potato proteins or potato starch. This film has a property called Gibbs film (Gibbs, 1961). This means in detail, that a viscous flow through a vertical film takes place in such a way that the two film surfaces remain in principle motionless. Therefore, the liquid enriched with proteins moves up as if it is between two solid walls (Prins, 1988). Additionally, so-called coalescence in our foam column occurred, when N_2 gas diffused from smaller to larger bubbles or bubble walls have been ruptured. These phenomenona are desired to a certain extent and cause reflux of protein enriched liquid enhancing the protein adsorption. A lot of experiments, taking the above-described procedures into consideration for an optimized foam-forming process, resulted in the operational conditions described in the section „Materials and Methods“.
- With initial protein concentrations of 0.5-0.9 g L^{-1} in the liquid pool the foaming process could be optimized.
- A further elongation of our foam column (> 1 m) did not influence the yield of concentrated proteins, but caused a channeling in the rising foam, which has to be avoided because of the consequent reduction of the protein amount in foamate.
- An addition of surfactants (e.g. starch, cationic and anionic agents), used over a wide range of pH (3-11) did effect neither protein enrichment nor separation and purification of protein mixture.
- The gas flow was also experimentally optimized and with a rate of 500 ml min^{-1} we were able to produce a foam of low-liquid consistence in our laboratory plant.
- Only spargers (glass frits) with a porosity of 3 were appropriate to form N_2 bubbles of a nearly uniform and optimal size.

But now back to the afore-mentioned unfolding of proteins in stress situations during foaming. In our continuous I-ABS experiments a part of the foamate was returned to the foam column in half height to serve as additional reflux. The repeated foaming and foam breaking of our potato protein solution ultimately causes denaturation. Therefore, this method does not appear to be useful when active proteins (enzymes) are involved.

The state of a protein at a surface/interface also depends on the concentration. The unfolded state of nearly all protein molecules occurs in diluted films, whereas concentrated films may contain only native proteins and unfolded molecules or molecules with different degrees of unfolding (Charm, 1972). These unfolding effects and loss of bioactivity were of subordinate or no importance in our experiments under the aspect, primarily, to generate a low-cost method for enrichment and isolation of potato protein fractions, which may deserve as a source of high nutritional food after removal from potato wastewater.

Under optimized foaming conditions in our laboratory scale foaming plant, a nearly fourfold improvement in protein concentration could be achieved as compared with nearly twofold in the batchwise operation.

A new cognition of these experiments is the pH-dependent, selective enrichment of defined protein fractions from a complex matrix like potato juice.

Potatoes possess proteins with an allergenic potential between pI = 4.5-5.2 (Wahl et al., 1990). With I-ABS it is possible, either to enrich the allergenic proteins in the foam and get a hypoallergenic solution, or to enrich the non-allergenic protein fractions in the foam and use the protein-enriched solution in the field of food industry for high-quality hypoallergenic nutrition.

The transferability to other complex protein solutions will be shown in further investigations.

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VIBRIO CHOLERAE ISOLATION METHOD IMPROVEMENTS IN ENVIRONMENTAL SAMPLES

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SUMMARY

Some methodological modifications are reported to isolate *Vibrio cholerae* from environmental samples, particularly marine water. Taurocholate addition in enrichment broth, higher incubation temperature in enrichment or isolation steps, string test and haemolytic activity as first confirmatory assays, increase the performance of standard procedures used for the detection of *Vibrio cholerae* in environmental specimens also if competitive microbial flora is present.

KEYWORDS: *Vibrio cholerae*, isolation, improvement of method, environmental samples

INTRODUCTION

The determination of *V. cholerae* in environmental samples is possible even in the absence of an evident fecal excretion [1]. *V. cholerae* non-01 is detected more frequently than *V. cholerae cholerae* in endemic regions in non epidemic periods [2 - 6]. However, isolation of cholerogenic *V. cholerae* 01 (*V. cholerare cholerae* 01) and 0139 serogroups [7] from environmental samples is difficult even during cholera epidemies, like in India or Bangladesh [8-10]. This difficulty was also experienced during the 1973's cholera epidemy in Italy [11] and later in South America (1991-1992) [12].

Factors responsible for the difficulty in isolation of *V. cholerae* 01 are essentially two: "stressed strains of *V. cholerae"*, not actively reproducing on artificial substrates [13] and low selectivity-electivity of isolation methods in the presence of competitive bacterial flora. In fact, on thiosulphate citrate bile sucrose (TCBS) agar various vibrios, different from *V. cholerae* (*Vibrio alginolatus, Vibrio fluvialis, Vibrio furnissii*) may show similar chromatic reactions (yellow colonies) [14].

Temperature, pH, dissolved oxygen, UV radiation, etc. affects *V. cholerae* when it is released in the environment. These conditions retard the duplication rate till *V. cholerae* enters in a latency phase that, under extreme conditions, induces dormancy and "viable but not cultivable" (VBNC) forms [13].

V. cholerae non-01 bacteria that infect only humans and other primates are prevalent in environment [15] because they survive better than *V. cholerae* 01, owing to their enhanced resistance to chemical pollutants [16].

In spite of these uncertainties, rapid isolation methods are required because of the clinical and epidemiological relevance of *V. cholerae.* A standard method was proposed by WHO [17].

The present paper suggests modifications to standard procedures, in order to increase the specific isolation of *V. cholerae* in environmental samples.

This research was carried out in the context of a surveillance program, set out in the province of Latina, Central Italy, following the death of a chronic liver patient from sepsis of doubtful origin.

MATERIALS AND METHODS

Water samples were collected aseptically from sewage, rivers, seawaters, in large neck bottles dipped just below the water surface.

A volume of water, 500 to 1000 ml, was filtered on membranes (FM) of glass fibre (1.2 µm) and cellulose nitrate (0.45 µm). The FMs were incubated in flasks containing 80-100 ml of APW (Alkaline Peptone Water) or APTW (Alkaline Peptone Taurocholate Water) initially at 36 ± 1 °C for 18 h (WHO [17]). After the incubation, a loop of the enrichment broth was streaked on Petri dishes containing TCBS (Thiosulphate Citrate Bile Sucrose) agar [18] or Monsur agar [17], incubated at 36 ± 1 °C for 18-24 hrs.

The colonies presumptively appearing as *V. cholerae* were isolated on Nutrient Agar (NA) slants, incubated at 36 ± 1 °C for 24 hrs and then assayed for oxidase test, catalase test, string test and finally Gram stained. The string test is a useful screening assay, especially proposed when antisera are not available [17]. On a glass microscope slide a loop of the test strain, grown on NA, is suspended in a drop of 0.5% aqueous desoxicholate solution; in this solution *V. cholerae* cells are lysed and form a mucoid string when a bacteriological loop is gently withdrawn from the suspension. The test differentiates *V. cholerae* from other environmental vibrios (*Vibrio parahaemolyticus*), but is not able to distinguish *V. cholerae* 01 from *V. cholerae* non-01.

Oxidase +, catalase +, Gram -, string test + -strains were streaked on sheep blood (5-10%) agar Petri dishes to evidence their haemolytic activity. After an aerobic incubation at 36 ± 1 °C for 18-24 hrs, the large translucent colonies, with clear ß-haemolysis areas around them, were tested with antisera.

V. cholerae strains so ascertained, were further confirmed by the following tests: motility, reaction in triple sugar iron agar (TSI), lysine-ornithinedecarboxylase, arginine-dihydrolase, sugar fermentation, O/F test, growth at increasing concentrations of NaCl (4-7 %) [17, 19, 20].

RESULTS AND DISCUSSION

Samples of surface waters (Table 1) were collected in the province of Latina and tested for the presence of *V. cholerae.*

The frequent detection of *V. cholerae* in wastewaters (30% of specimens) and in freshwaters (60% of specimens) could not be confirmed in isolation experiments for the determination of *V. cholerae* in marine environment. One of the possible reasons may be due to the over growth of *V. alainolyticus* in saltwaters. This finding indicates the need to increase the selectivity of the entire procedure. Temperature was found to be the major selective factor for *V. cholerae* isolation. By increasing the incubation temperature from 36 ± 1 to 42 ± 0.5 °C, *V. cholerae* strains began to appear also in seawater samples. Out of 20 marine water samples, 5 were analyzed according to the Standard methodology proposed by WHO (incubation temperature 36 ± 1 °C) [17, 20], while the remaining were incubated at 42 ± 0.5 °C (Table 2). The 80 % efficiency rate in *V. cholerae* isolation, achieved with the introduction of higher incubation temperatures (42 ± 0.5 °C) in the enrichment step (Table 2), is considerably high for envirorimental samples rich in antagonistic microbial flora.

The low salt contents of enrichment and selective media proved to be another discriminating factor in increasing selectivity. Growth assays were performed using a strain of *V. alginolyticus*, isolated during this same survey programme (Table 3).

At high incubation temperatures *V. alginolyticus* becomes strictly halophylic, while being facultatively halophylic at 36 ± 1 °C.

The incubation temperature of 42 ± 0.5 °C, critical in the enrichment step, may be too selective in the isolation step: using 42 ± 0.5 °C as incubation temperature also in this phase, no colonies could be developed on the selective agarized media.

TABLE 1 - Isolation of *V. cholerae* in environmental samples, using standard procedure [17].

Samples	Total tested samples	Positive samples
Wastewaters	30	9
Rivers	10	6
Seawater	5	0

TABLE 2 - Strains isolated from seawater samples and confirmed as *V. cholerae* (Temperatures are referred to enrichment step).

Samples/ Temperature	Presumptive *Vibrio Cholerae*	Confirmed *Vibrio Choleraae*
36 ± 1 °C	5	0 (0%)
42 ± 0.5 °C	15	12 (80%)

TABLE 3 - Growth (+) of *Vibrio alginolyticus* on TCBS (Thiosulphate Citrate Bile Sucrose) and NA (Nutrient Agar) with different temperatures and salinities.

Media	Salinities (NaCl, %)	Temperature (° C) 36 ±1	42 ± 0.5
TCBS	1	+	-
NA	0.5	+	-
NA	4	+	+
NA	7	+	+

In the enrichment step, at least for marine water samples, APTW gave better results than APW in the isolation of *V. cholerae.* The following step represented by streaking loops of enrichment broth on TCBS agar or Monsur agar has indicated that the first agarized medium was better for selective purposes and easier to use by operators than the second one.

This research was stimulated by the observation that it is difficult, if not impossible, to detect *V. cholerae* in marine environment even when it is present and detectable in tributary rivers and in sewage wastewaters dumping into the sea. This fact seems difficult to explain, considering the well-known alophylic properties of *V. cholerae.* It should be considered as an artefact due to improper isolation in thods and high competition of other environmental bacteria, many of them belonging to the same family of Vibrionaceae.

High temperatures (41 ± 0.5 °C) are recommended by WHO as selective for enrichment of enterotoxic *V. parahaemolyticus* [21], but not for the isolation on TCBS agar. In this last case the indicated temperature is 36 ± 1 °C [17, 18, 21].

The beneficial selective effect of temperature increase in the isolation of *V. cholerae* from environmental samples was previously reported by De Paola et al. [22], in particular, for some difficult specimens, as shellfish homogenates [23]. For testing marine water samples, the temperature of 42 ± 0.5 °C, applied in the enrichment step using APTW, increased yields and made easier to isolate *V. cholerae*, even in the presence of other environmental Vibrionaceae (i.e. *V. alginolyticus* [24]. The increased temperature (from 36 ± 1 !C to 42 ± 0.5 °C) favours not only the isolation of *V. cholerae*, but also PCR detection of cholera toxin gene [25, 26].

Other evidences collected during this research can be summarized as follows:

- Taurocholate in the enrichment broth increases the selective revitalisation of *V. cholerae* [17]
- Monsur agar, compared to TCBS agar, has several disadvantages: presumptive *V. cholerae* colonies are not easily distinguished for their growth patterns. Moreover, the substrate, not on sale, is rather complicated to prepare in laboratory.
- The most effective tests for primary confirmation of *V. cholerae* are string test [17] and haemolytic activity. However, haemolysis can be temporary, defective in environmental isolates of choleratoxic *V. cholerae* (containing the ctx gene) [27]. Non-haemolytic strains can regain the haemolytical activity [28]. This feature belongs to the typical mutability of *V. cholerae*, that affects growth capacity [13], seroconversion [29 -32] and gene expression [33, 34].

The strains isolated in the present work were all related to *V. cholerae* non-01 and NAG vibrios which may have an epidemiological relevance in environmental samples [35]. However, the sensitivity of a methodology is appropriate when all *V. cholerae* species can be isolated in environmental samples.

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PRODUCTION OF CELLULASES BY BACILLUS SUBTILIS STRAINS CULTURED ON WASTEWATER

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SUMMARY

Bacteria isolated from hot springs in Zimbabwe were screened for the production of endo-β-1,4-glucanase enzyme using the Congo red method. Six endo- β-1,4-glucanase producing bacterial strains were identified as belonging to the genus *Bacillus.* The bacteria produced endo- β-1,4-xylanase and protease enzymes when cultured on nutrient rich media in batch cultures. The six strains were also able to produce endo-β-1,4-glucanase, endo-β-1,4-xylanase and protease enzymes when cultured using starch processing industrial waste water and yeast culture waste water in batch culture. In continuous cultures, where starch processing waste water was used, α- amylase was produced in addition to the above-mentioned enzymes. The bacteria have the potential to be used for the production of hydrolytic enzymes or for the treatment of industrial wastes.

KEYWORDS:
Cellulase, hydrolyitic enzyme, waste water, *Bacillus*

INTRODUCTION

The production of cellulolytic enzymes by bacteria has been observed very often [1-5]. The complete hydrolysis of cellulose requires several jointly acting enzymes such as cellulases, endoglucanases, exoglucanases (cellobiohydrolases) and ß-glucosidase [6-12]. There have been many studies on bacterial cellulases [13-15]. Many bacteria of the genus *Bacillus* exclusively produce endo-β-1,4-glucanase, an endocellulase, belonging to the group of cellulases [16–19]. Some members of the genus *Bacillus* produce not only endoglucanase but also many other extracellular enzymes such as protease, xylanase and amylase [20].

Main fields of applications of cellulases at present are bio-polishing of fabrics and production of stonewashed denims in textile industry or usage as detergents to make fabrics soft and bright in household laundry [21]. But cellulases are also applicated in baking industry, deinking of paper, clarification of fruit juices and improving the nutritional quality and digestibility of animal feed [22]. A major potential area of application of cellulases is the bioconversion of renewable cellulosic biomass to commodity chemicals [14, 23 - 26].

Hot springs are a good source of microorganisms producing, especially, thermostable enzymes for industrial applications. A thermostable enzyme is also likely to be stable against denaturants that unfold its tertiary structure. Working at high temperatures has a number of advantages: higher reaction rates, inhibition of microbial growth, increasing solubilities of some substrates, and decreasing viscosity of solutions [27].

Our laboratory has been interested in studying the conditions of endoglucanase production by *Bacillus* strains over the past few years [19, 28, 29]. In this study we isolated several bacterial strains from local hot springs and screened them for the production of endo-β-1,4-glucanase using both batch cultures and continuous culture systems. Additionally, the production of other hydrolytic enzymes by the endoglucanase producing bacteria was investigated. In the study it was also tried out to use food-related wastewaters as media for growth and enzyme production by these strains.

MATERIALS AND METHODS

Isolation

One hundred and twelve bacterial strains were isolated from hot springs at three sites in Zimbabwe:

- Chimanimani, wells with 44 °C, 45 °C and 55 °C, all at pH 9.0
- Chiredzi, one well at 42 °C with pH 7.5 and
- Binga, wells with 40 °C, 55 °C and 96 °C, all at pH 8.0.

The bacteria were isolated from hot spring water using Hutner mineral base medium [30] containing 10 g l^{-1} carboxymethlycellulose (CMC) as the carbon source. The bacterial strains isolated from the hot spring water were then screened for the production of cellulase by streaking out on modified Degryse *et al.* [31] medium agar plates containing (g l^{-1}): bactotryptone (Difco) 2.5; yeast extract (Difco) 2.5; carboxymethlycellulose (CMC) 10.0; agar (Difco) 20, minerals and phospate buffer 0.05 mol l^{-1}. The medium had a pH of 7. Bacterial colonies showing cellulolytic activity were selected by flooding the plates with 0.1% aqueous Congo Red. The dye was decanted after 20 min and the plates were flooded with 5M NaCl. After 20-30 min the NaCl was decanted and endogluca-nase producing colonies were identified as those surrounded by a clear zone against a red background [9, 32].

The endoglucanase producing strains were analysed for sporulation, catalase motility and Gram reaction. The API 50 CHB system was used for 49 physiological tests, according to the manufacturer's instructions (bioMerieux, France). API strips were inoculated with bacteria from 12 h modified Degryse *et al.* medium agar plates suspended in API 50 CHB medium. The API strips were incubated at 40 °C for 18 h.

Enzyme production in batch cultures

The isolates were cultured in modified Degryse synthetic medium with 10 g l^{-1} CMC as the carbon source. Filter paper activity in the cultures was investigated by growing the strains in modified Dergyse medium and Hutner mineral base medium with crystalline cellulose, Avicel PH-101 (Fluka) and Whatman Filter paper No. 1 at 10 g l^{-1}, as the carbon sources.

The strains were also cultured on wastewater used in the production of starch from maize and wastewater from the production of yeast biomass from molasses. No additional nutrients were added to the wastewater media. The pH of the medium was adjusted to 6.0, the optimum pH for endoglucanase production for one of the bacterial strains [19]. Citrate-phospate buffer 0.05 mol l^{-1} was added to the media which were sterilized for 15 min at 121 °C. All the shake flask experiments were conducted in 500 ml Erlenmeyer flasks with a working volume of 200 ml and incubated at 200 rev min^{-1}. The cultures were maintained at 50 °C, the optimum temperature for two of the strains [19]. Samples were taken at intervals of 24 h and the supernatants were kept frozen at -20 °C until analysis.

Enzyme production in continuous cultures

Strain HR68 was cultured in continuous culture using starch waste water. The pH of the waste water was adjusted to 6.0 and buffer was added at 0.05 mol l^{-1}. The medium was sterilized for 15 min at 121°C.

The culture was run in a 2 litre Applikon Fermentor with a working volume of 1 l. The cultures were run at dilution rates of 0.03 h^{-1} and 0.08 h^{-1}. Sampling was done at steady state and hydrolytic enzymes were determined as for the batch culture samples.

Analysis of samples

The determination of endo-β-1,4-glucanase activity was done according to the IUPAC recommended method [33]. Activity of the enzyme was assayed by incubating 200 µl of culture supernatant with 1.8 ml of 1% hydroxyethylcellulose (HEC) in citrate-phosphate buffer (pH 6.0, 0.05 mol l^{-1}) at 60 °C for 10 min. The reducing sugars produced were measured by the 3,5-dinitrosalicylic reagent method (DNS) [34].

Filter paper activity for the determination of exocellulase enzyme activity was measured according to the IUPAC recommended method [33] by incubating Whatman No.1 filter paper strips (1.0 cm x 6.0 cm) in 1.0 ml of citrate-phosphate buffer (pH 6.0, 0.05 mol l^{-1}) with 0.5 ml of culture supernatant at 60 °C for 1 h. The reducing sugars produced were measured by the aforementioned DNS reagent method [34].

Cellobiase enzyme activity was measured according to the IUPAC recommended method [33]. 1.0 ml of culture supernatant was incubated with 1.0 ml of 15.0 mM cellobiose in citrate-phosphate buffer (pH 6.0, 0.05 mol l^{-1}) at 60 °C for 1 h. The glucose produced was measured using a glucose oxidase reaction kit (Boehringer Mannheim, Germany).

Protease activity was determined by incubating 1.0 ml of culture supernatant with 1.0 ml of 0.5% (w/v) azocasein in citrate-phosphate buffer (pH 6, 0.2 mol l^{-1}) at 60 °C for 1 h. The reaction was stopped by adding 2.0 ml of 10% (w/v) trichloroacetic acid [35] The mixture was then centrifuged at 6,000 g for 10 min and the absorbance measured at 380 nm. The activity of the protease was expressed in abitrary units (1 U is equivalent to an OD 380 change of 0.001 nm min^{-1}).

Xylanase activity was measured according to the method of Bailey *et al*. [36] by incubating 200 µl of culture supernatant with 1.8 ml of 1% xylan in citrate-phosphate buffer (pH6.0, 0.05M) at 60 °C for 10 min. The concentration of reducing sugars produced was determined by the DNS reagent method [34].

Amylase activity was measured using the method of Giraud *et al.* [37]. 100 ml of culture supernatant was incubated with 0.8 ml of 1.2% soluble starch (pH6.0, 0.05M) at 60 °C for 10 min. The reducing sugars produced were also analyzed by the DNS reagent method [34]. All assays were done in triplicate.

TABLE 1 - Endoglucanase, xylanase, amylase and protease enzyme production (maximum amount) by *Bacillus* isolates cultured on modified Degryse medium at 50° C in batch culture.

isolate	Endoglucanase (nkat ml-1)	Xylanase (nkat ml-1)	Protease (Units ml^{-1})	Amylase (µmol $ml^{-1}min^{-1}$)
CH32	81	82	ND	ND
CH43	83	59	2.8	1.5
HR68	137	74	4.5	4.5
HR77	125	51	6.9	1.4
HR80	95	59	23.2	4.5
HR86	120	60	9.5	0.5

ND = not detected

TABLE 2 - Endoglucanase, xylanase and protease enzyme production (maximum amount) by *Bacillus* isolates cultured on buffered starch waste water at 50 °C in batch culture.

isolate	Endoglucanase (nkat ml-1)	Xylanase (nkat ml-1)	Protease (Units ml^{-1})	Amylase (µmol $ml^{-1}min^{-1}$)
CH32	68	25	0.1	ND
CH43	53	43	0.1	ND
HR68	63	51	0.2	ND
HR77	84	54	0.2	ND
HR80	60	64	0.1	ND
HR86	101	47	0.1	ND

ND = not detected

TABLE 3 - Endoglucanase, xylanase and protease enzyme production (maximum amount) by *Bacillus* isolates cultured on yeast culture waste water at 50° C in batch culture.

isolate	Endoglucanase (nkat ml-1)	Xylanase (nkat ml-1)	Protease (Units ml^{-1})	Amylase (µmol $ml^{-1}min^{-1}$)
CH32	65	55	0.4	ND
CH43	72	50	0.4	ND
HR68	81	57	0.2	ND
HR77	70	51	ND	ND
HR80	63	49	3.0	ND
HR86	67	42	0.4	ND

ND = not detected

TABLE 4 - Endoglucanase, xylanase, protease and amylase enzyme production by *Bacillus* isolate HR68 continuously cultured on starch wastewater at 50 °C.

Dilution Rate	Endoglucanase (nkat ml-1)	Xylanase (nkat ml-1)	Protease (Units ml^{-1})	Amylase (µmol $ml^{-1}min^{-1}$)
0.03	40	47	0.4	1.7
0.08	8	51	0.3	1.5

RESULTS AND DISCUSSION

Isolation and characterisation

A total of hundred and twelve bacterial strains were isolated from the hot springs, of these 12 showed endoglucanase production when screened using Congo red dye. Using submerged cultures, 6 strains, CH32 and CH43 from Chiredzi hot springs and HR68, HR77, HR80 and HR86 from Chimanimani hot springs, evidenced appreciable endoglucanase production, which were chosen for identification and further enzyme production studies.

All the six bacterial strains were Gram-positive, obligate aerobic rods that readily form endospores. Furthermore, they all were catalase positive utilizing a wide range of carbon sources as shown by analysis with the API 50 CHB system. The bacteria were identified as members of the genus *Bacillus.* The API results suggested that the strains were closely related to the *B. subtilis* group. *Bacillus* species that are considered a part of the *B. subtilis* group *include B. subtilis, B. pumulis, B. licheniformis, B. megaterium* and *B. amyloliquefaciens* [38, 39]. *Bacillus* species are heterogeneous with some not well defined until now. There also exist varieties between strains of the same species [39, 40].

Additional biochemical tests showed that the bacteria were Voges-Proskauer positive, utilize starch for growth and produce urease confirming their identification as strains of the *Bacillus subtilis* group [38, 41]. Moreover, the identity of these bacteria was reaffirmed by the Germany Collection of Microorganisms (DSM).

Enzyme production in batch cultures

When cultured on modified Degryse *et al.* medium, all the strains produced endo-β-1,4-glucanase, but no filter paper or cellobiase activity was found. All strains, except CH32, produced also xylanase, α-amylase and protease when cultured on modified Degryse *et al.* medium. Strain CH32 produced only xylanase (Table 1).

When cultured on starch wastewater or on wastewater from the production of yeast biomass, all the strains produced endoglucanase, xylanase and protease, but no amylase (Tables 2 and 3). Strain HR77 did not produce protease on the yeast culture waste.

In all cultivations it was observed that the pH of the cultures increased, which may be due to the formation of alkaline products during growth. The yield of endoglucanase and xylanase by all strains was generally lower when cultured on wastewater. Protease and amylase were produced in low amounts or not at all in the wastewater cultures.

Enzyme production in continuous culture

Endoglucanase, xylanase, protease and α-amylase were produced by the strain HR68 in continuous culture (Table 4). Filter paper and cellobiase activity were not detected at all in these cultures. Endoglucanase, protease and α-amylase activity was higher at a dilution rate of 0.03 h^{-1}, while the xylanase activity was higher at 0.08 h^{-1}. The hydrolytic enzymes produced by strain HR68 in continuous cultures could be applied in continuous culture waste treatment [42].

The results obtained with all the other strains under examination suggest that production of the enzymes is predominantly influenced by nutrient availability. Endoglucanase and xylanase production do not appear to be as sensitive to nutrient conditions as protease and α-amylase productions. The wastewater could, therefore, be used as medium only for the production of endoglucanase and xylanase by the *Bacillus* strains.

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POLYUNSATURATED FATTY ACIDS OF GREEN AND BROWN ALGAE FROM THE EGYPTIAN MEDITERRANEAN COAST

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SUMMARY

Five species of green algae belonging to the two orders Ulvales and Bryopsidales in addition to five species of brown algae belonging to the orders Fucales, Dictyotales and Scytosiphonales were screened for their polyunsaturated fatty acids (PUFAs) according to seasonal variations and different locations from the Mediterranean Sea in Alexandria. The Ulvales (*Ulva lactuca, Ulva fasciata* and *Enteromorpha intestinalis*) were characterized by highest levels of $C_{16:4}$ (n-3) and $C_{18:4}$ (n-3) reaching to (15.65 %) for $C_{16:4}$ (n-3) in *Enteromorpha intestinalis* in autumn and 24.9 % for $C_{18:4}$ (n-3) in *Ulva lactuca* in summer. The ratio of $C_{16:4}$ (n-3) to $_{16::3}$ (n-3) was more than one. The Bryopsidales (*Caulerpa racemosa* and *Codium repens*) were characterized by high level of $C_{16::3}$ (n-3) (11.5 %) with low levels of $C_{16:\ 4}$ (n-3) and $C_{18:\ 4}$ (n-3).

As for the brown algae, the dominant PUFAs were C_{18} and C_{20}. The percentage of arachidonic acid (AA) $C_{20:4}$ (n-3) was higher than that of eicosapentaenoic acid (EPA) $_{20:5}$ (n-3). Highest level of AA was 38.14 % in *Sargassum hornschuchii* (Fucales) in spring. Maximum docosahexaenoic acid DHA $C_{22:6}$ (n-3) (8.5 %) was detected in *Dictyota dichotoma* (Dictyotales) in spring.

KEYWORDS: Algae, omega 3 fatty acids (EPA, DHA), polyun-saturated acids (PUFAs), arachidonic acid (AA)

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) especially those with 20 carbon atoms are currently receiving attention in view of their physiological, industrial and pharmaceutical values. These acids cause lowering of cholesterol and triglycerols in plasma, prevention of atherosclerosis and other cardiovascular diseases and reduction of collagen-induced thrombocyte aggregation [1]. In addition, these acids are used for the biosynthesis of eicosanoid hormones [1, 2]. They are of interest in biotechnology and more recently in cosmetics [3, 4].

In addition, doco-sahexaenoic acid (DHA) is one of the most abundant components of the brains structural lipids and the lipid fraction of human mother`s milk contains thirty times of the level of DHA observed in cows` milk lipid. DHA may be important in the development of brain tissue of babies [5]. Marine plants are recognized producers of C_{18} and C_{20} PUFAs. For this reason, considerable interest among researchers was aroused [6, 7]. Studies of Phaeophyta fatty acids have not so far been systemic although brown algae are the dominant flora of many temperate littoral zones[8].

In a recent research in Germany a new type of eggs enriched with Omega 3 fatty acids was produced [9] which regulates blood circulatory system [10]. Our earlier work addressed the PUFAs in some red macrophytic algae in the Mediterranean Sea in Egypt [11]. The present paper reports further results on PUFAs of 10 brown and green algae from the same sea.

MATERIALS AND METHODS

Algae: The green macrophytic algae *Codium repens*, *Caulerpa racemosa*, *Enteromorpha intestinalis*, *Ulva lactuca*, *Ulva fasciata* and the brown macrophytic algae *Colpomenia sinuosa*, *Dictyota dichotoma*, *Petalonia fascia*, *Sargassum hornschuchii* and *Zonaria flava* (see Table 1) were collected at different seasons and different locations from the shores of Alexandria (see Table 2), thoroughly cleaned and washed in distilled water.

Lipid separation: Freeze-dried samples of algae were extracted with chloroform / methanol according to the method of Bligh and Dyer [12]. Lipids were applied to silica gel-120 (Merck) column (1.5 x 30 cm). The samples were first purified by elution with 100 ml petroleum ether (40-60^{o} C), then the glyceride fraction was collected using 200 ml of dried benzene. The benzene fraction was dried by passing over anhydrous sodium sulphate; then the solvent was evaporated by vacuum under nitrogen.

Lipid transmethylation:
Freeze-dried samples were methylated using 0.5 M sodium methoxide solution in methanol [13]. Heptadecanoic acid was added as an internal standard. The methylated samples were tested for complete estrification by TLC using silica gel-60 (Merck). The developing solvent used was petroleum ether / ether / acetic acid (90/ 10/ 1) [14].

Fatty acid analysis:
Fatty acids were determined by gas liquid chromatography of the methyl esters. Methyl esters were analysed with a Helwett Packard 5880 gas chromatograph equipped with a flame ionization detector and a fused silica capillary column (25 m x 0.25 mm) coated with Carbowax 20 M. Operating conditions were: temperature program 100-230^{o} C, rate of temperature increase 8 ^{o}C min^{-1} for 5 min.

The carrier gas was nitrogen with a flow rate of 1 ml min^{-1}, with a split ratio of 1/ 20. Fatty acid methyl esters were identified by co-chromatography with authentic standards (Sigma Chemical Co., St. Louis, MO) and by GC-MS analysis performed with a Finnigan Mat SSQ 7000 mass spectrometer equipped with a DB-Wax capillary column (30 m x 0.25 mm) under the following operation conditions: isothermal for 3 min at 100 ^{o}C, temperature program was 100-230 ^{o}C at 8 ^{o}C min^{-1} rate, then isothermal at 230 ^{o}C for 40 min. Chemical ionization spectra were obtained at EI 70 eV.

The quantity of the fatty acids was determined by comparing their peak areas with that of the internal standard. The data shown are the mean values of at least two independent samples, each analyzed in duplicate.

TABLE 1 - Taxonomic relationship of the algae studied

No.	Division	Class	Order	Family	Species
1	Chlorophycota	Chlorophyceae	Ulvales	Ulvaceae	*Ulva lactuca*
2					*Ulva fasciata*
3					*Enteromorpha intestinalis*
4			Bryopsidales	Caulerpaceae	*Caulerpa racemosa*
5				Codiaceae	*Codium repens*
6	Chromophycota	Phaeophyceae	Scytosiphonales	Scytosiphonaceae	*Colpomenia sinuosa*
7					*Petalonia fascia*
8			Dictyotales	Dictyotaceae	*Dictyota dichotoma*
9					*Zonaria flava*
10			Fucales	Sargassaceae	*Sargassum hornschuchii*

RESULTS AND DISCUSSION

The results in Table 2 show the locations and time of collection of the ten Chlorophycota and Chromophycota species, in addition to their total lipids. As for the 5 green algal species, the total lipids ranged from 2.1 % in *Codium repens* to 5.6 % in *Ulva fasciata* in Abu-Kheer and Stanley, respectively, in winter. The total lipids of the other three species ranged from 2.2 % in *Ulva lactuca* in summer to 3.86 % in *Enteromorpha intestinalis* in autumn in Montaza and Miami shores. Highest lipid content in the brown algae was detected in *Dictyota dichotoma* which ranged from 6.44 % in winter in Stanley shore to 9.0 % in spring in Miami shore. The total lipids in *Petalonia fascia* was more or less similar in spring and winter (6.39 % - 6.6 %) in Miami and Ras- Elteen shore, respectively. The lowest lipid contents were detected in *Colpomenia sinuosa* and *Sargassum hornschuchii* (2 % and 2.2 %, respectively) in spring in Miami shores.

The unsaturated fatty acids (PUFAs) composition as presented in mol. percent (GC analysis) of the ten Chlorophycota and Chromophycota species is reported in Table 3. The two polytetraenoic acids $_{16:4}$ (n-3) and $_{18:4}$ (n-3) were dominant in the order Ulvales (*Ulva lactuca, Ulva fasciata* and *Enteromorpha intestinalis)*. Maximum levels of these two acids were 15.65 % and 24.9 % in *Enteromorpha intestinalis* and *Ulva lactuca* in autumn and summer, respectively. These results are similar to those reported for other algae of this phylum [15, 16, 17, 18]. In addition, the ratio of $_{16:4}$ (n-3) to $_{16:3}$ (n-3) is more than one in the three mentioned species, which is also similar to those reported by Johns et al. [19] and Jamieson and Reid [20]. In this respect, it was mentioned by Dembitsky et al. [21] that the green algal species from the Black Sea were unusual in containing $_{16:4}$ (n-3) varying from 4.9 to 23.1 % of the total fatty acids which is in agreement with

our results. The results also indicated the dominance of $_{18:3}$ (n-3) and $_{18:2}$ (n-3) which are similar to those mentioned for Ulvales from the Senegalese coast [22]. The high levels of $_{18:4}$ (n-3) distinguish the Ulvales from Cladophorales [22]. As for the two green algae *Codium repens* and *Caulerpa racemosa* (order Bryopsidales), the dominant PUFA was $_{16:3}$ (n-3), while the two tetraenoic acids $_{16:4}$ (n-3) and $_{18:4}$ (n-3) were found in low levels. This was mentioned to be the most important chemotaxonomic character of the order Bryopsidales [18, 23]. In this respect, it was also mentioned by Klenk et al. [24] and Sato [25] that $_{16:3}$ (n-3) is a characteristic PUFA for *Codium* species. The DHA $_{22:6}$ (n-3) accounted for 4.7 % - 5.7 % in *Ulva lactuca* and *Ulva fasciata* in summer in Stanley and Miami shores, respectively. In addition, arachidonic acid (AA) $_{20:4}$ (n-6) (8.28 %) was detected in *Ulva fasciata* in summer in Miami shores. The PUFAs C_{20} and C_{22} were found only in smaller amounts in the five green algal species than in the Chromophycota or Rhodophyta [20].

As for the five brown algal species, it can be seen that C18 and C20 PUFAs were the major fatty acids which made up more than 50% of the total fatty acids. This is typical of all brown algae [6, 16, 20, 22]. In the majority of Chromophycota species, the percentage of arachidonic acid AA $_{20:4}$ (n-6) is higher than that of eicosapentaenoic acid (EPA) $_{20:5}$ (n-3). In this respect, it was mentioned by Fleurence et al. [26] that brown algae had high contents of $_{18:3}$ (n-3), but low content of $_{20:5}$ (n-3).

In *Sargassum hornschuchii*, arachidonic acid AA (38.4 %) predominated among polyenoic acids which is in accordance with the results mentioned before that AA predominated in all *Sargassum* species examined except *S. miyabei* [27]. AA has been reported to be a major component FAs in some *Sargassum* species [28]. The DHA level in the five brown algae ranged from 2.4 % in *Zonaria flava* (in summer) in Abu-kheer shore to 8.5 % in *Dictyota dichotoma* (in spring) in Miami shore.

From the results it can also be seen that no much difference was noted between the PUFAs contents in different seasons and different locations in the five screened brown algae. This is in contrast to what was mentioned before for the four Rhodophyta species [11]. Up to our knowledge, no work was published on this topic in the Egyptian shores of the Mediterranean.

TABLE 2 - Total lipids and time of collection of the investigated algae

No.	Time of collection	Area of collection	Total lipids (%)
1	Spring 97	Montaza	3.43
	Summer 97	Montaza	2.24
	Summer 98	Stanley	3.38
	Summer 98	Ras-Elteen	2.70
	Autumn 98	Miami	3.13
	Autumn 98	Gleem	3.84
2	Summer 1998	Miami	2.80
	Winter 1999	Montaza	3.34
	Winter 1999	Miami	3.35
	Winter 1999	Stanley	5.60
	Winter 1999	Ras-Elteen	4.62
3	Summer 1998	Miami	3.51
	Autumn 1998	Montaza	3.37
	Autumn 1998	Miami	3.86
	Autumn 1998	Gleem	3.03
	Autumn 1998	Stanley	3.66
4	Summer 1997	Montaza	3.47
5	Winter 1997	Abu-Kheer	2.10
	Summer 1997	Abu-Kheer	2.90
	Autumn 1997	Abu-Kheer	2.30
6	Spring 1999	Miami	2.00
7	Winter 1999	Ras-Elteen	6.60
	Spring 1999	Miami	6.39
8	Winter 1999	Stanley	6.44
	Spring 1999	Miami	9.00
	Spring 1999	Stanley	6.72
9	Summer 1997	Abu-Kheer	4.63
10	Spring 1999	Miami	2.22

TABLE 3 - Fatty acids composition in Chlorophycota and Chromophycota species.

Algae No.	Area of collection	Time of Collection	Fatty acids %													
			C16 :0	C16 :1	C16:2	C16:3	C16:4	C18:0	C18:1	C18:2	C18:3	C18:4	C20:4	C20:5	C22:6	Others
1	Montaza	Spring 97	23.50	0.90	1.20	1.30	7.10	3.10	10.21	9.50	16.00	12.00	1.10	1.56	-ve	12.53
	Montaza	Summer 97	13.50	1.70	1.90	0.90	9.86	4.15	5.95	10.75	15.20	14.50	0.95	1.10	-ve	19.55
	Stanly	Summer 98	8.70	tr	tr	tr	4.88	tr	5.00	10.20	21.00	23.60	0.60	-ve	4.70	21.32
	Ras-Elteen	Summer 98	4.90	tr	tr	tr	9.99	2.25	-ve	10.00	25.80	24.90	2.10	-ve	2.00	18.06
	Miami	Autumn 98	4.25	1.74	1.40	1.20	5.10	1.02	1.30	16.70	20.30	19.20	1.25	1.22	-ve	25.30
	Gleem	Autumn 98	4.30	2.70	0.66	0.58	7.20	0.70	1.90	11.60	22.80	16.10	-ve	2.20	-ve	29.26
	Montaza	Autumn 98	8.00	1.66	0.75	1.03	8.10	0.80	2.30	13.57	21.76	17.10	-ve	3.10	-ve	21.83
2	Miami	Summer 98	6.70	3.10	tr	tr	-ve	tr	-ve	17.90	25.70	14.07	8.28	-ve	5.70	18.55
	Stanley	Winter 99	7.70	0.90	1.00	0.90	9.50	1.20	2.10	6.10	9.00	13.10	0.50	1.50	-ve	46.5
	Montaza	Winter 99	7.10	0.80	1.10	0.80	9.40	1.10	2.00	5.89	8.88	13.30	0.40	1.30	-ve	47.93
	Miami	Winter 99	7.60	0.90	1.00	0.90	9.30	1.10	2.05	5.79	8.78	12.90	0.35	1.23	-ve	48.10
	Ras-Elteen	Winter 99	8.10	0.88	1.10	0.89	9.20	1.00	1.99	5.80	8.30	13.01	0.38	1.31	-ve	31.04
3	Miami	Summer 98	28.00	1.90	1.58	2.57	7.60	1.60	7.90	8.70	20.38	12.70	-ve	0.90	0.80	5.37
	Montaza	Autumn 98	28.20	0.37	0.34	3.60	15.65	0.90	5.46	8.80	4.10	17.10	3.30	0.76	0.33	11.09
	Miami	Autumn 98	24.60	0.50	2.60	2.40	13.40	0.80	4.66	7.20	2.90	12.70	2.60	0.70	-ve	24.90
	Gleem	Autumn 98	25.00	0.90	2.10	1.10	12.00	1.10	4.60	8.30	1.10	6.10	2.10	0.80	-ve	34.80
	Stanley	Autumn 98	22.70	0.80	1.87	4.03	14.27	0.80	3.66	4.20	1.46	10.70	2.30	0.65	-ve	32.56
4	Montaza	Summer 97	16.00	0.60	0.50	11.0	0.30	0.50	4.00	11.00	22.00	1.70	1.80	-ve	0.70	29.90
5	Abu-kheer	Winter 97	9.30	0.50	tr	5.60	tr	1.50	3.34	14.30	36.30	1.70	tr	tr	2.90	24.60
	Abu-kheer	Summer 97	31.70	0.80	2.70	11.4	tr	1.60	8.90	12.60	11.60	3.20	4.15	0.80	4.00	6.55
	Abu-kheer	Autumn 97	26.50	0.70	2.10	11.5	tr	1.10	15.90	9.80	14.10	1.50	1.00	2.10	tr	13.70
6	Miami	Spring 99	14.00	2.70	0.10	0.10	-ve	-ve	15.10	4.10	7.80	10.70	21.90	8.70	4.70	10.10
	Ras-Elteen	Winter 99	16.40	2.40	0.10	0.10	-ve	-ve	14.20	5.50	4.30	8.60	18.64	9.90	4.50	15.40
	Miami	Winter 99	15.89	2.30	0.10	0.10	-ve	-ve	13.90	5.45	4.10	8.58	17.22	8.90	5.50	17.96
7	Ras-Elteen	Winter 99	2.30	-ve	2.40	-ve	5.28	3.13	2.19	3.69	5.79	-ve	17.30	21.9	5.68	30.30
	Miami	Winter 99	3.50	-ve	1.40	-ve	6.72	3.20	2.90	4.00	6.17	-ve	18.40	17.9	4.50	31.30
	Miami	Spring 99	3.40	-ve	1.30	-ve	5.78	2.80	2.89	3.90	6.00	-ve	17.30	16.5	4.20	35.93
8	Stanley	Winter 99	5.27	1.00	0.80	1.50	-ve	-ve	15.28	10.49	7.40	9.13	14.40	5.38	4.95	24.40
	Miami	Spring 99	8.30	1.70	2.90	1.73	-ve	-ve	7.49	1.14	9.40	6.65	14.26	6.56	8.50	31.40
	Stanley	Spring 99	5.90	0.90	0.80	2.30	-ve	-ve	16.80	0.93	8.20	10.00	13.60	6.40	4.10	30.10
9	Abu-kheer	Summer 97	24.90	1.26	0.17	-ve	-ve	0.60	17.90	6.07	11.80	7.10	4.30	2.90	2.40	20.60
10	Miami	Spring 99	3.67	0.90	1.10	-ve	-ve	6.10	3.50	1.99	4.42	-ve	38.14	5.90	1.94	32.30

tr = traces; -ve = detectable

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MICROBIOLOGICAL AND CHEMICAL QUALITY OF VACUUM-PACKED SMOKED SALMON UP TO THE DECLARED EXPIRY DATE

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SUMMARY

Thirty-two samples of vacuum-packed smoked salmon from various places were analyzed from the microbiological, chemical and physical point of view at the declared expiry date. Microbiological and chemical results obtained evidenced that hygienic quality of the smoked salmon at the expiry date was very poor and that only a small number of samples could be kept for more than 30 days. Standardization of production technology, adoption of microbiological limits, parameters for a_w and sodium chloride and the prevention of the use of bacterial starters are extremely important to guarantee the quality of vacuum-packed smoked salmon.

KEYWORDS: Smoked salmon, shelf-life, vacuum-packed

INTRODUCTION

Vacuum-packed sliced smoked salmon is highly sought after and consumed worldwide during periods of celebration, but also on a daily basis. The reasons for the increase in demand are probably linked to the fact that the smoked salmon fully satisfies not only hedonistic standards but also those of the modern diet, as a product with simple organoleptic qualities, that is easy to prepare and of high dietetic and nutritive value.

The manufacturing companies that produce smoked salmon, have subjected the raw material to rather low-level technological conservation procedures (salting, carried out using small amounts of salt; smoking, performed at low temperature and for a short time) in order to give the end product the organoleptic qualities required. On the one hand, this mild technology has allowed the introduction on the market of a widely approved product, but on the other hand, it has raised hygienic and sanitary doubts [1].

Since the preservative action of the salt and smoke is very mild, the product's shelf-life is mainly linked to the storage temperature. In fact, when this technology is used, only refrigeration at 0 °C would control the bacterial growth and early alteration of the product [2].

Current laws, which do not establish the microbiological standards for smoked salmon, give the companies the chance to introduce into the market a product with variable limits (30 to 90 days) with regard to the expiry date.

The aim of the present survey was to evaluate either the quality or the state of preservation of the smoked salmon up to the expiry date declared by the manufacturer and to focus on the hygienic and organoleptic characteristics of the product.

MATERIALS AND METHODS

Thirty-two packs of smoked and vacuum-packed salmon from Norway, Scotland, Denmark and Ireland were purchased from local shops and analyzed. All of the packs were held at 4°C until the expiry date recommended by the manufacturer, since we believed this was the most common situation during marketing and domestic storage.

Sensory analysis. The packs were evaluated for integrity, swelling and presence of liquid. Organoleptic examinations (colour, odour and texture) of the product were carried out either immediately after opening or a few minutes later.

Microbiological analysis. Twenty grams of the specimens were homogenized for 1 min in a Stomacher device (PBI International, Italy) after adding 180 ml of sterile physiological saline. Then, they were ten-fold diluted.

Aerobic mesophylic bacterial count:
1 ml of each dilution was included in Plate Count Agar (OXOID, England) and incubated at 25 °C for 48 h in duplicate.

Psychrophilic bacterial count:
0.1 ml of each dilution was plated on Plate Count Agar (OXOID, England) and incubated at 2-4 °C for 8-10 days in duplicate.

Enterobacterial count:
1 ml of each dilution was plated on Violet Red Bile Agar (VRBA) (OXOID, England) supplemented with 1% glucose. The plates were incubated in duplicate at 32 °C for 24-48 h.

Total coliform count:
1 ml of each dilution was plated on VRBA (OXOID, England). The plates were incubated in duplicate at 37 °C for 24-48 h.

Lactobacillus spp count:
10 grams of samples were homogenized in 90 ml of Ringer solution; the homogenate was used to prepare ten-fold dilutions in MRS broth (OXOID, England); 1 ml of each dilution was included in plates of MRS agar (OXOID, England). The incubation was carried out in jars at 25 °C for 72 h and anaerobiosis was achieved by means of a "Gas Generating " kit (OXOID, England).

Chemical and physical analysis. The water content was determined by means of the infra-red electronic analyzer MA 30-000V2 (SARTORIUS, Germany). Water activity was determined using the "AW ECO" (PBI international, Italy), consisting of a sensor able to detect and measure the amount of a_w in the sample. The chloride ions were measured according to Pearson by using a silver nitrate standard solution and potassium chromate as an indicator [3]. The percentages of water content and sodium chloride obtained were correlated to each other, in order to determine the preservation index (P.I.) applying the following formula [1]:

$$\text{P.I.} = \frac{[\text{NaCl (\%)} \times 100]}{\text{Water content}}$$

Chromatography was used to determine biogenic amines (histamine, putrescine, cadaverine, spermine and spermidine), as suggested by Mietz *et al.* [4]. The biogenic amine/polyamine index (B.A.I.) was calculated using the following equation [4]:

$$\text{B.A.I.} = \frac{(\text{ppm histamine} + \text{ppm putrescine} + \text{ppm cadaverine})}{(1 + \text{ppm putescine} + \text{ppm spermidine})}$$

RESULTS AND DISCUSSION

Labeling - The labels of 53% of the samples examined did not show the date of production. Therefore, calculating the real duration of storage period suggested by the manufacturer was impossible. By law (D.L. 109/92; Community Directive 89/395; Community Directive 89/396) [5], the manufacturer is not obliged to reveal on the label the date of production. In some cases, it is explicit in the bar code. The manufacturing companies had given the shelf-life as 30, 45, 60, 75, and 90 days, respectively, for 16%, 3%, 19%, 3%, and 6% of the samples examined. Information about the storage temperature was widely different (0 °C to +2 °C, 0 °C to +4 °C, 0 °C to +5 °C).

Organoleptic examination. None of the packs examined revealed any alteration such as swelling or presence of liquid. At the opening of most packs a pungent smell was perceived but it quickly vanished. One sample revealed a strong smell of putrefaction and the texture of the fillets was less firm. The colour of the products examined varied from pink to orange and some samples revealed portions of tissue of anomalous colours probably due to minor trauma or technological defects.

Bacteriological results. The results of bacteriological investigations are reported in Table 1. The samples examined showed extremely variable aerobic mesophilic bacterial counts ($<10^5$ cfu g^{-1} to 10^8 cfu g^{-1}); in most cases (90.6%) the values ranged between 10^7 to 10^8 cfu g^{-1}. These data are in accordance with those reported in the literature [6, 7, 8]. Psychrophilic bacteria revealed, in 34.4% of the cases, values as high as the mesophylic counts, differing from these only by 1 or 2 lower logarithmical powers; this difference was only higher for two samples. Also, the *Lactobacillus* spp. count was the same as the aerobic mesophilic bacteria, confirming that almost the whole of the vacuum-packed smoked salmon bacterial flora at the expiry date consisted of microorganisms belonging to this group, as described by Huss [9]. Only one sample revealed aerobic mesophilic bacterial values as high as 10^8 and very low levels of lactic bacteria (3.4 x 10 cfu/g). In this sample the count of enterobacteria and coliforms was proportionally higher (10^7 cfu/g). This microbiological situation is the explanation for the poor organoleptic characteristics revealed at the moment of opening the pack. The enterobacterial counts of the other samples examined were extremely variable and as high as 10 cfu/g in 25 % of the samples, 10 and 10^2 cfu/g in 9.3 %, 10^2 and 10^3, and 10^3 and 10^5 cfu/g in 15.6% of the samples and higher than 10^5 cfu/g in 18.7% of the samples. With regard to the total coliform count, the results obtained were the same as those described for the enterobacteria; 37% of the samples revealed values less than 10 cfu/g; 12.5% ranged between 10 and 10^2, 9.3% between 10^2 and 10^3, and 12.5% between 10^3 and 10^5. The same percentage was obtained for those samples whose bacterial count was higher than 10^5 cfu/g.

TABLE 1 - Results of microbiological analysis (cfu g^{-1})

Sample	Aerobes	Psychrophils	Enterobacteria	Coliforms	Lactic bacteria
1	$3x10^8$	$6,1x10^5$	$<10^4$	$<10^4$	$4x10^8$
2	$4x10^8$	$1,1x10^7$	$<10^4$	$<10^4$	$6x10^9$
3	$2,2x10^8$	$4,4x10^6$	$<10^4$	$<10^4$	$7x10^8$
4	$1,8x10^8$	$8,8x10^6$	$1,8x10^7$	$1,4x10^7$	3,4x10
5	$6x10^7$	$5,5x10^6$	$<10^4$	$<10^4$	$1,6x10^8$
6	$3,8x10^7$	$3,2x10^6$	$2x10^6$	$1x10^6$	$8x10^7$
7	$2,8x10^6$	10^5	$<10^4$	$<10^4$	$1,1x10^8$
8	$4,7x10^7$	$4,8x10^6$	$4x10^6$	$2,2x10^6$	$1,2x10^8$
9	$3,3x10^8$	$7x10^6$	$4x10^6$	$4,6x10^4$	$2,2x10^8$
10	$1,5x10^7$	$8,3x10^6$	<10	<10	$1,3x10^7$
11	$2x10^8$	$5x10^6$	$4,7x10^3$	$1x10^3$	$2,4x10^8$
12	$1,2x10^8$	$5,4x10^6$	10^3	$7x10^2$	$1,5x10^8$
13	$2,1x10^8$	$1x10^8$	<10	<10	$2,6x10^8$
14	$1,7x10^8$	$7,6x10^7$	1x10	<10	$2,1x10^8$
15	$7,6x10^8$	$1,3x10^8$	7x10	6x10	$4x10^8$
16	$3,8x10^7$	$2x10^6$	$8,1x10^2$	<10	$1,8x10^8$
17	$8,3x10^7$	$8,1x10^7$	<10	<10	$3,1x10^8$
18	$4,6x10^6$	$5,8x10^6$	<10	<10	N.D.*
19	$7,6x10^7$	$7x10^7$	$3,2x10^3$	$3,2x10^3$	$1,1x10^8$
20	$2,7x10^8$	$1,6x10^8$	$7,6x10^4$	$5,1x10^4$	$4,8x10^8$
21	$1,6x10^7$	$2,3x10^7$	<10	<10	$2,4x10^7$
22	$1,2x10^8$	$1,6x10^7$	$1,6x10^4$	1x10	$2,1x10^8$
23	$1,5x10^8$	$3x10^7$	$1,1x10^2$	7x10	$3,6x10^8$
24	$4,6x10^7$	$3x10^7$	<10	<10	$2,1x10^8$
25	$2,3x10^7$	$2.6x10^7$	$3,2x10^6$	$6,4x10^4$	$2,2x10^7$
26	$5,5x10^7$	$3x10^7$	$3,7x10^3$	<10	$1,7x10^7$
27	$1,3x10^8$	$2,5x10^7$	$8,1x10^6$	$1,9x10^6$	$8,1x10^7$
28	$3,7x10^8$	$4x10^7$	<10	<10	$3.1x10^8$
29	$<10^5$	10^5	<10	<10	$<10^5$
30	$1,4x10^7$	10^5	$3x10^2$	$1,4x10^2$	$3,6x10^7$
31	$8,3x10^7$	10^8	1x10	<10	$4,8x10^8$
32	$5,6x10^8$	$5,5x10^4$	$3x10^2$	6x10	$1x10^9$

*not determined

TABLE 2 - Results of chemical and physical analysis

Samples	a_w	Water %	NaCl %	P.I.	B.A.I
1	0.96	60.5	4.2	7	13,2
2	0.95	65.6	2	3.1	9,9
3	0.97	60.9	3.7	6.1	26,8
4	0.97	59.7	2.3	3.8	46
5	0.96	60.9	3.4	5.6	84
6	0.95	65.7	3	4.5	10,7
7	0.96	54.6	4.8	8.8	48,6
8	0.96	58.1	2.5	4.3	18,3
9	0.96	63.8	2.4	3.7	8,7
10	0.95	58	3.1	5.4	25
11	0.95	58.2	1.9	3.3	14,2
12	0.97	57.1	2	3.5	15,3
13	0.95	70.4	2.9	4.1	18,3
14	0.95	61.6	2.8	4.5	11,3
15	0.97	62.7	2.9	4.6	14,2
16	0.96	59.6	2.1	3.6	8,8
17	0.97	46	1.4	3.1	9,4
18	0.97	61	2.5	4.1	7,2
19	0.97	67.8	1.3	1.9	13,1
20	0.93	60	1.7	2.9	9,8
21	0.96	41.1	5.2	12.7	8,7
22	0.96	62.9	3.2	5.1	11,5
23	0.94	59.2	2.7	4.6	32,4
24	0.96	50.8	4.6	9	6,8
25	0.96	61	3	5	16,4
26	0.96	56.7	3.3	5.9	21,4
27	0.95	63	1.4	2.3	18,7
28	0.95	60.7	3.8	6.2	5,8
29	0.95	59.6	4.8	8	6,1
30	0.95	63.7	3.8	6	16,7
31	0.97	59.8	1.2	2	9,2
32	0.96	64.2	3.8	6	10,8

P.I. = preservation index; B.A.I. = biogenic amine/polyamine index

Results of the chemical and physical analysis.

The results [water content, sodium chloride content, preservation index and water activity (a_w)] of the chemical and physical analysis are reported in Table 2: All the samples contained <5% sodium chloride, which is well-known not to guarantee the hygienic and sanitary qualities of the salmon, as previously described by Cantoni [10].

The water content of 6 % of the samples was higher than 67 % and ranged between 60 and 67 % in 62 % of the samples. This range is believed to be, by Cantoni [1], the upper limit for a product shelf-life longer than 30 days.

Therefore, the results obtained show that only a few of the samples examined can actually be kept for more than 30 days.

The results concerning the water activity are very similar in all the samples examined and ranged between 0.95 and 0.97. These values, firstly, reveal the mild preservation treatment applied to the product and, secondly, represent the major cause of bacterial growth.

B.A.I. (biogenic amine/polyamine index) values (Table 2) led us to consider that 34 % of the samples examined had ideal hygienic characteristics, whereas those samples whose index was higher than 10 were considered to be altered, even when not revealing any change when organoleptically examined. Most of the samples whose B.A.I. was higher than 10 had been claimed by the manufacturing companies to have a longer shelf-life (60-90 days). Nevertheless, some other samples with a shorter shelf-life (30 days) revealed B.A.I. values very close or slightly higher than the limit value [10, 11].

The microbiological and chemical results obtained by the present survey show that the hygienic quality of the smoked salmon packs analyzed at the expiry date is very poor. The hygiene of smoked salmon depends on different factors. The most important one is the extremely variable microbiological conditions of the raw material, that is often severely contaminated by psychrophilic flora. Other parameters contribute to determining the hygienic quality of the end product: the observance of the Good Manufacturing Practice (GMP), the use of techonology suitable to guarantee the reduction of the psychrophilic flora and, most of all, the storage temperature of the end product.

During the distribution and the sale of the product, as well as during domestic storage, the preservation temperature usually used ranges between 4 and 6 °C [8, 12, 13].

It is well-known [1, 6, 7] that, in order to prevent any decomposition of the smoked salmon, a temperature between 0 and 1 °C should be maintained. In fact, maintenance of this temperature can prevent the growth of psychrophilic microorganisms present in the raw material and not completely eliminated by the mild preservation treatment.

The preservation temperatures indicated on the label of the samples examined prove that, despite the prolonged expiry date, the manufacturing companies are perfectly aware of the possibility that hygienic and sanitary problems might arise. In fact, in many cases, the temperatures suggested are very difficult to achieve during the transport and sale of the product and, above all, during domestic storage (0 - 2°C). It is to be hoped that the manufacturing companies define the shelf-life taking into consideration the storage conditions as closely as possible to the real ones (4 - 6°C).

Our survey did not reveal any precise correlation between bacterial count, preservation index (P.I.) and presence of biogenic amines.

The B.A.I. of most samples indicates a mild or severe alteration of the product [9, 14, 15], which was not revealed by organoleptic or microbiological examination. It is possible to assert that this index, successfully used for food preservations to evaluate the precise hygienic quality of the raw material used, might not be the right qualitative parameter for smoked salmon, considering the fact that even technologically useful *Lactobacillus* spp. [16] can present decarboxylation activity.

We believe it to be more useful to establish the sanitary aspect of the product by defining the highest tolerable concentration of biogenic amines in the end product as the critical limit.

In conclusion, adoption of microbiological limits at the moment of sale, the definition of the shelf-life at temperatures ranging from 4 °C to 6 °C, the standardization of production technology with the adoption of parameters for a_w and sodium chloride and the prevention of the use of bacterial starters are extremely important to guarantee the quality of vaccum-packed smoked salmon products.

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EINE NEUE GC-MS METHODE ZUR BESTIMMUNG VON SAFROL UND CUMARIN IN GEWÜRZEN, AROMEN UND EXTRAKTEN

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ZUSAMMENFASSUNG

Die Änderung der EG-Aromenrichtlinie 88/388/EEC wird derzeit beraten. In dieser Richtlinie werden u.a. neue Grenzwerte für den Gehalt von Safrol (0,5 bzw. 0,05 mg/kg) und Cumarin (0,5 mg/kg) in Lebensmitteln und Getränken festgelegt. Der vorliegende Artikel beschreibt eine neue GC-MS SIM-Methode zur Bestimmung von Safrol und Cumarin in Gewürzen, Aromen und Extrakten. Die Ergebnisse der Analysen werden in Hinblick auf die Anwendung der neuen Aromenrichtlinie diskutiert.

KEYWORDS: GC-MS, SIM, Analyse, Cumarin, Safrol, Zimt, Muskatnuss, Macis, Grenzwerte, Lebensmittel, Getränk, etherisches Öl, Extrakte, Gewürz, Aroma.

EINLEITUNG

Der Europarat berät derzeit eine Änderung der EG-Aromenrichtlinie 88/388/EEC [1]. Diese Richtlinie behandelt, neben einer Vielzahl anderer Substanzen, auch zulässige Gehalte von Safrol und Cumarin, die als Inhaltsstoffe in Gewürzen wie Zimt, Muskat und Macis vorkommen [2-6]. Als neue Grenzwerte für Cumarin werden u.a. 0,5 mg/kg (ppm) in Lebensmitteln (Foodstuffs) und Getränken (Beverages) diskutiert. Gemäß der neuen Richtlinie darf der Gehalt von Safrol in Lebensmitteln 0.5 mg/kg und in Getränken 0.05 mg/kg nicht übersteigen. Höhere Grenzwerte für Safrol sind nur zulässig im Falle von alkoholischen Getränken (0,5 mg/kg)) sowie Lebensmitteln mit primärem Macis- oder Muskatnuß-Aroma (15 mg/kg).

Die neue Richtlinie greift jedoch nicht, wenn den Lebensmitteln zwar Gewürze, aber keine Aromen zugesetzt wurden. Beispielsweise wird eine ausschließlich mit gemahlenem Zimt gewürzte Backware auch dann als verkehrsfähig eingestuft, wenn der Cumarin-Gehalt den Grenzwert überschreitet. Wurde bei der Herstellung jedoch zusätzlich ein cumarinhaltiges Zimtaroma verwendet, gelten die diskutierten Höchstmengen. Analoges gilt für Safrol-Gehalte in Lebensmitteln bei dem Einsatz von Muskat und Macis.

Der vorliegende Artikel beschreibt eine neue Methode zur gemeinsamen, analytischen Bestimmung von Cumarin und Safrol in Gewürzen, sowie aus daraus hergestellten aromatisierenden Produkten wie Wasserdampfdestillaten (etherischen Ölen) und Hochdruckextrakten. Die im Rahmen der Untersuchung dieser Produkte gewonnenen Daten werden hinsichtlich der Konsequenzen diskutiert, die sich aus der neuen EU-Richtlinie ergeben.

MATERIAL UND METHODEN

Probenbeschreibung

Als „Canehl“ werden Proben von ceylonesischem Zimt (*Cinnamomum zeylanicum* - Blume), als „Cassia“ werden Proben von chinesischem Zimt (*Cinnamomum cassia* - Blume) bezeichnet. Bei der Muskatnuß handelt es sich um den Samen von *Myristica fragrans* Houtt., als Macis wird die Samenhaut (auch Blüte) von *Myristica fragrans* Houtt. bezeichnet. Bei allen untersuchten Produkten handelt es sich um in Deutschland handelsübliche Gewürze und Aromen.

Bestimmung von Cumarin und Safrol in verschiedenen Proben

1.GC-MS

Zum Nachweis von Cumarin und Safrol kann die gleiche GC-MS Methode verwendet werden. Die Quantifizierung der Verbindungen erfolgt mit der Methode des Internen Standards (100% Methode), wobei folgende Substanzen als Standards verwendet werden:

Interne Standards (IS):

(1) *Cumaranon* als IS für Safrol (IS 1)
(2) *3,4-Methylendioxy-phenyl-acetonitril* als IS für Cumarin (IS 2)
(3) *Decansäuremethylester* zur Kontrolle der Wiederfindung der IS

- GC-MS Parameter

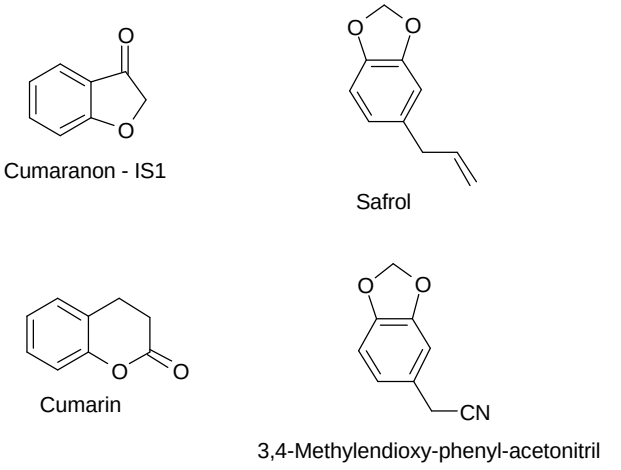
Hewlett-Packard 5890 Series II Gaschromatograph
Temperaturprogramm: 100°(5) –2°–> 144° –20°–>250°(20)↓
Injektion: 1 µl, mit Split (1:20)
Trägergas: He, Vordruck: 0,6 bar
Injektor: Siemens Split/Splitlos; 230 °C
Säule: J&W, DB 05ms, 30m × 0,25mm, 0,25µm Filmdicke
Detektor: HP 5971 Series MSD

Hewlett-Packard 5971 Series MSD
Temperatur, Transferline: 285° C
Temperatur, Ionenquelle: 195° C
Ionisationsmodus: EI, 70 eV
TIC-Messungen: Scan: 45 bis 200 u Scan/second: 2,8

SIM-Messungen:

Verbindung	Zeit [min]	m/z
1 IS 1	5 – 11	134, 105
2 Safrol	11 – 14	162
3 IS 3	14 – 18	87, 74
4 Cumarin	18 – 22	146, 118
5 IS 2	22 - Ende	161, 160

ABBILDUNG. 1: Safrol, Cumarin und die internen Standards, die zur quantitativen Analyse benötigt werden



Aufarbeitung

Wasserdampfdestillate :

0,45 g etherisches Öl

\+ 1 ml einer Lösung, die die internen Standards (IS) 1 und 2 in der Konzentration 0,5 mg/ml in Ethylacetat enthält mit Ethylacetat auffüllen auf 5 ml. Die Lösung ist fertig zum Einspritzen.

Die Zugabe des dritten Internen Standards entfällt, da die Aufarbeitung ausschließlich aus einem Verdünnungsschritt besteht.

Rohzimt, -macis, -muskat:

Die Proben werden unter mehrfachem Übergiessen mit flüssigem Stickstoff in einem Mörser zu einem möglichst feinen Pulver zermahlen. Das so erhaltene Pulver wurde über ein Sieb (Maschenweite: 300 µm) ausgesiebt und der Feinanteil zur Analyse verwendet.

0,5 – 3 g Pulver, je nach Cumarin- bzw. Safrolgehalt

→ Soxhlet-Extraktion mit 200 ml Ethylacetat für drei Stunden. Vor Beginn der Extraktion werden dem Löungsmittel 2 ml einer Lösung, die die internen Standards 1+2 zu je 0,1 mg/ml enthält, zugesetzt. Ebenfalls vor der Extraktion werden die Kühler der Soxhlet-Extraktoren mit Hilfe eines Kryostaten auf 5° C temperiert. Nach beendeter Extraktion werden die Kühler zusätzlich mit 50 ml Ethylacetat durchspült. Die Spüllösung wird mit dem Extrakt vereinigt.

Das Lösungsmittel wird über eine 30 cm lange Vigreux-Kolonne mit Claissen-Destillationsbrücke abdestilliert, bis der Extrakt auf ca. 3ml eingeengt ist. Anschliessend werden 50 µl einer Lösung, die den internen Standard 3 in der Konzentration 4 mg/ml in Ethylacetat enthält, hinzugefügt. Die so erhaltene Lösung kann zur qualitativen und quantitativen Analyse direkt in das GC-MS-System eingespritzt werden.

Sollte sich die Lösung trüben, kann zunächst versucht werden, die ausgefallenen Bestandteile durch Zugabe von Ethylacetat bis zu 6 ml in Lösung zu bringen. Falls die Lösung anschliessend immer noch trübe ist, sollte die Probe nach beendeter Niederschlagsbildung etwa 1 min in ein Ultraschallbad gehalten und anschliessend membranfiltriert (0,45µm) werden.

Wiederfindungsraten gegenüber IS (3):

IS (1): 90%; IS (2): 96%

HD-Extrakte von Macis und Muskat:

0,05 – 0,1g Extrakt

→ auf etwas Watte in Soxhlet-Hülse einwiegen und weiter aufarbeiten wie die Pulver, jedoch die doppelte Menge an internen Standards zusetzen und bei der abschliessenden Destillation nur bis zu 6 ml einengen.

Wiederfindungsraten gegenüber IS (3):

IS (1): 90%; IS (2): 96%

Durchführung der Wasserdampfdestillation

10g des Zimtpulvers werden in einen 250 ml Einhalskolben eingewogen. Der Kolben wird anschliessend zur Hälfte mit Wasser gefüllt und über eine 20 cm lange Vigreux-Kolonne an eine Claissen-Destillationsbrücke mit Wasserkühlung angeschlossen. Als Vorlage wird ein 100 ml Ein-halskolben verwendet. Als Heizquelle für die Destillation dient ein 250 ml Heizpilz. Nach einsetzender Kondensation des Produktes in der Vorlage wird die Destillation noch zwei Stunden fortgeführt. Anschliessend wird das Öl von der wässrigen Phase des Destillates mittels eines 100 ml Scheidetrichters abgetrennt.

ERGEBNISSE UND DISKUSSION

Methodische Ergebnisse

Die etherischen Öle wurden zur Analyse verdünnt und nach Zugabe der internen Standards direkt in das GC-MS eingespritzt. Die HD-Extrakte lösten sich schlecht in den gängigen Lösungsmitteln und waren aufgrund ihres Gehalts an Fetten, Wachsen und freien Fettsäuren zur direkten Injektion in das GC-MS System nicht geeignet. Um in der zur Verfügung stehenden Zeit eine adäquate Aufarbeitungsmethode bereit zu stellen, wurden die HD-Extrakte in der gleichen Weise behandelt wie die Rohwaren. Die Entwicklung einer Gelpermeationschromatographie (GPC)–Methode zur Abtrennung der Triglyceride und freien Fettsäuren von den Aromabestandteilen wird für die Zukunft jedoch anvisiert.

Zur Extraktion der homogenisierten und pulverisierten Rohwaren erschien die Soxhlet-Extraktion am besten geeignet. Die anfangs insbesondere für Cumaranon schlechte Wiederfindungsrate konnte durch den Einsatz eines Kryostaten (Temperierung der Rückflusskühler vor Beginn der Extraktion auf 5 °C) stark verbessert werden. Leichtsiedende Verbindungen, die sich nach Ablauf der Extraktionsdauer von drei Stunden eventuell im Kühler oder im Soxhlet-Aufsatz befinden, werden durch das Spülen der Apparatur mit ca. 50 ml Ethylacetat in den Kolben zurückgebracht.

Auf diese Weise wurden Wiederfindungsraten von 90 ± 5 %für Cumaranon und 96 ± 4 % für den Internen Standard 3,4-Methylendioxy-phenyl-acetonitril erreicht (n = 6). Aufgrund der vergleichbaren Flüchtigkeiten von Cumaranon und Safrol sowie 3,4-Methylendioxy-phenyl-acetonitril und Cumarin, wird von einer vergleichbaren Wiederfindungsrate von Analyt und zugeordnetem internem Standard im Verlauf einer Analyse ausgegangen. Die Wiederfindungsrate der internen Standards wurde für jede Analyse mit Hilfe eines dritten internen Standards kontrolliert. Durch diese Vorgehensweise kann von einer hohen Sicherheit in Bezug auf die erarbeiteten Daten ausgegangen werden. Neben der Flüchtigkeit der Analyten und internen Standards wurde das Ergebnis der Analysen zusätzlich durch den Vermahlungsgrad der Rohwaren beeinflußt. Zimt ist eine Rinde, die aufgrund ihrer Zähigkeit schwer zu vermahlen ist. Übergiesst man die Rinde mehrfach mit flüssigem Stickstoff, so wird sie spröde und läßt sich in einem Mörser per Hand vermahlen. Die Analyse kann nur dann ein richtiges Ergebnis liefern, wenn vor der Soxleth-Extraktion sichergestellt wird, daß die Zellstruktur der Pflanzen vollständig aufgebrochen ist. Bei Zimtrinden war das der Fall, nachdem das handgemahlene Pulver durch ein Sieb der Maschenweite 300 µm von gröberen Mahlgutbestandteilen abgetrennt wurde. Auch bei Muskat und Macis wurden durch die Verwendung feinerer Siebe keine Anhebung der Analysenresultate mehr beobachtet.

Ergebnisse der Untersuchung von Zimt

In keiner der untersuchten Zimtproben konnte Safrol nachgewiesen werden, obwohl in der Literatur Safrol als Inhaltsstoff des Ceylonesischen Zimtes erwähnt wird [2]. Hinsichtlich des Cumarin-Gehaltes unterschieden sich die beiden untersuchten Zimtsorten auffällig. Während im Ceylonesischen Zimt durchweg nur geringe Cumaringehalte festgestellt wurden, enthielt der Chinesische "Cassia"-Zimt durchweg sehr hohe Mengen davon. Diese Ergebnisse stehen im Einklang mit den Angaben in der Literatur [3-5].

Chinesischer Cassia-Zimt wird aufgrund seines hohen Gehaltes an Zimtaldehyd und des damit verbundenen intensiven Geschmacks gerne in Lebensmitteln eingesetzt. Legt man einen Cumarin-Gehalt im Cassia von nur 1000 ppm zugrunde, dann wird der von der EU-Kommission für Aromen angestrebte Grenzwert durch das Gewürz bereits bei einer Zugabe von 0.05 Gew.% zum Lebensmittel überschritten. Die Rezeptur für ein handelsübliches, mit Zimt gewürztes Müsli kann schon die zehnfache Menge, also 0,5 % Zimt enthalten. Bei Verwendung von ceylonesischem Canehl-Zimt kann der Grenzwert für Cumarin auch bei einem Zimtgehalt von 0,5 Gew. % im Lebensmittel garantiert werden. Aufgrund seines Gehaltes an Eugenol und einiger anderer Phenylpropane weist diese Zimtsorte jedoch ein anderes Aromaprofil als der Cassia auf.

ABB. 2 - Korngrößenverteilung der untersuchten Zimtproben – durch Sieben wird sichergestellt, daß nur Partikel mit aufgebrochener Zellstruktur zur Analyse kommen

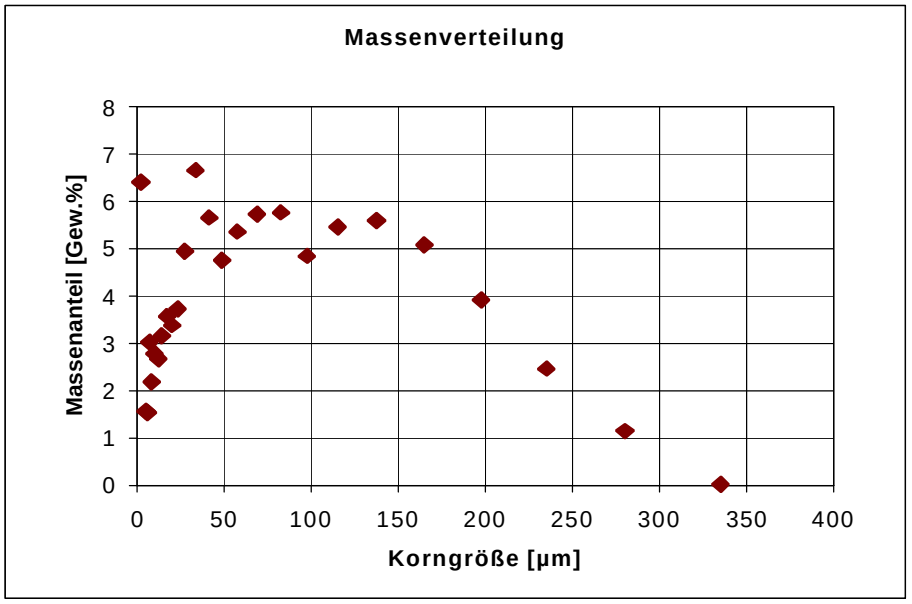


TABELLE 1 - Ergebnistabelle nach Untersuchung verschiedener handelsüblicher Zimt-Rohwaren.

Canehl-Sorten	Cumarin-Gehalt [ppm]
Sri Lanka	8
Sri Lanka Quillings 101	31
Sri Lanka Quillings 109	9
Sri Lanka Pure Quillings	14
Sri Lanka Superfine	9

Cassia-Sorten	
Cassia 1	825
Cassia 2	3260
Cassia 3	2725

TABELLE 2 - Ergebnistabelle nach Untersuchung verschiedener handelsüblicher Muskat- und Macisprodukte

Macis Produkte	*Safrol-Gehalt [ppm]*
gemahlene Rohware 1	3570
gemahlene Rohware 2	3560
Macis HD-Extrakt	1033
Macis Lösungsmittelextrakt	11460
Macis Wasserdampfdestillat	20950

Muskat Produkte	
gemahlene Rohware	242
Muskat HD-Extrakt	709

Soll im Rahmen einer freiwilligen Selbstkontrolle der Industrie der Cumarin-Grenzwert für ein zimthaltiges Produkt eingehalten werden, und ist die Verwendung von Cassia-Zimt zur Erlangung eines harmonischen Aromaprofils dennoch erwünscht, so besteht durch die Vorgabe der EU Kommission in der Praxis immer noch die Möglichkeit, beide Zimtsorten kontrolliert miteinander zu verschneiden.

Nach unseren Untersuchungen ist Cumarin zudem nur in geringem Umfang wasserdampfflüchtig. Ein aus 10 g Cassia-Zimt im Labor hergestelltes Destillat (Ausbeute: ca. 70 µl) enthielt neben dem Hauptbestandteil Zimtaldehyd lediglich **1247 ppm** Cumarin. Das gleichzeitig überdestillierte Wasser hatte einen Gehalt von 17,9 ppm Cumarin. Der Cumaringehalt der Rohware hatte bei 825 ppm gelegen. Von der gesamten, in der Probe enthaltenen Cumarinmenge ist also nur ca. der 100. Teil im etherischen Öl (Zimt-Aroma) enthalten. In einem zweiten Versuch wurden 10 g eines Cassia Zimtpulvers verwendet, das einen Cumaringehalt von 3260 ppm aufwies. Nach der Wasserdampfdestillation wurden wiederum 70 µl Produkt erhalten, das lediglich mit 1655 ppm Cumarin belastet war.

Diese Ergebnisse sollten in Zukunft noch durch die Analyse verschiedener handelsüblicher Wasserdampfdestillate abgesichert werden. Für die Praxis könnte diese Beobachtung jedoch bedeuten, daß Zimtaroma in Form des etherischen Öles an Stelle des Gewürzes eingesetzt, die Belastung des Lebensmittels mit Cumarin um zwei Größenordnungen absenkt. Demnach sollten sich die Grenzwerte der EU-Kommission auch mit aus natürlichem Zimt gewonnenen etherischen Ölen einhalten lassen.

Ergebnisse der Untersuchung von Muskat und Macis

Wie der Ergebnistabelle entnommen werden kann, weist die Macis einen wesentlich höheren Safrolgehalt auf, als die Muskatnuß. Aufgrund der geringen Probenzahl lässt sich nur vermuten, daß es sich dabei um eine ganze Größenordnung handelt. Bei safrolhaltigen Aromen spielt zudem die Verarbeitung eine außerordentlich große Rolle. Neben dem Wasserdampfdestillat stand bei dieser Untersuchung auch ein mit CO_2 als Lösungsmittel gewonnener Extrakt, ein sogenannter Hochdruck(HD)-Extrakt, zur Verfügung.

Im Fall von Safrol führt die Wasserdampfdestillation nicht zur Abreicherung, sondern zu einer Anreicherung der Substanz im Aroma. Die Verwendung von Wasserdampfdestillaten aus Muskat oder Macis als Aromen dient damit nicht der Entlastung des Endproduktes.

Beim HD-Extrakt hingegen konnte eine deutliche Abreicherung von Safrol im Aroma festgestellt werden. Offensichtlich ist Safrol nicht ausreichend in der unpolaren CO_2–Phase löslich, die hier zur Extraktion verwendet wird. Der Einsatz von HD-Produkten als Alternative zum Gewürz kann demzufolge zur Prüfung empfohlen werden. Zwar enthält der HD-Extrakt von Muskat, verglichen mit dem Gewürz, aus dem er hergestellt wurde, die dreifache Menge an Safrol. Es darf dabei jedoch nicht vergessen werden, daß es sich bei dem Extrakt um ein Konzentrat handelt, von dem nur ein Bruchteil des Gewichtes zum Würzen eingesetzt wird, das bei Verwendung der gemahlenen Rohware erforderlich wäre. Bei HD-Extrakten der Macis fällt diese Betrachtung noch wesentlich günstiger aus.

Beim Macis-Lösungsmittelextrakt handelt es sich um einen Auszug, der durch ein nicht näher beschriebenes Verfahren hergestellt wurde. Hinsichtlich des Safrol-Gehaltes nimmt dieser Lösungsmittelauszug eine mittlere Position zwischen Wasserdampfdestillat und HD-Extrakt ein.

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MINERAL AND PHYTATE CONTENTS OF 10 VEGETABLES GROWN IN NIGERIA AND CALCULATION OF THEIR PHYTATE:ZN AND CA:PHYTATE MOLAR RATIOS

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SUMMARY

10 different varieties of vegetables were evaluated for their mineral and phytate contents. The concentrations of Ca, K, Mg, and Na were found to be high, whereas Zn, Fe, and Mn were low. Cu was not detected at all. The levels of phytic acid (phytate) and phytate P were generally high and nearly all vegetables had more than 50% of their total phosphorus linked to phytic acid (myoinositol hexaphosphoric acid) partly occurring as an acid and partly as salts, such as the insoluble calcium phytate in plant foods. The phytate:Zn and Ca:phytate molar ratios for all varieties of vegetables analysed were relatively high. Because phytate readily forms compexes with minerals such as Ca and Zn, these results showed that with high phytate contents the bioavailability of minerals is relatively low.

KEYWORDS:
Mineral composition, phytate, molar ratio, vegetables

INTRODUCTION

Vegetables are essential in human diet. They not only contain proteins which are equal in quality to animal proteins but also are an important source of mineral elements especially calcium and iron as well as carbobydrates, fibre and vitamins [1-3]. In developing countries, vegetables are needed as a complementary mixture with other types of foodstuffs to produce a balanced diet. Vegetables are not only useful for human health but also for crop rotation to increase the soils` nitrogen status or for the canning industries, just to mention a few.

Phytic acid (phytate) is one of the allelocheminals found in foods and feeds [4]. It is nutritionally important because of the formation of complexes with calcium, magnesium, copper, and proteins [5], thereby making them unavailable for man and animals [6]. By competitive chelate formation phytate participates in the process of intestinal absorption of Ca, Mg and Fe ions.

This formation of insoluble chelate complexes of phytic acid accounts for some of its extremely important properties. Furthermore, phytic acid is the most important antinutritional factor (ANF) because more than 50% of P is present in the form of phytate in cereals, legumes, seeds and organic soils [7] serving as a phosphate depot in the body, which is broken down by phytase to myo-inositol (human body contains 40 g of myo-inositol). It also plays the role of a growth factor. Myo-inositol increases the oxygen transporting capacity of hemoglobin in red blood cells, improves and regulates cellular metabolism, especially, in conditions of phosphorus deficiency in the body, stimulates hemopoiesis and bone tissue formation, and also improves the tone of the nervous system [8].

Since phytates are associated with dietary fibre, the objectives of this study were to quantify the mineral phytate complexes and the phytate contents. On the basis of these results the molar ratios of phytate:Zn and Ca:phytate were calculated for vegetables of major consumption.

MATERIALS AND METHODS

The different varieties of vegetables were obtained from the Federal College of Agriculture Campus in Akure. The vegetables under investigation are shown in Table 1.

Processing methods

250 g of the vegetables were washed, sun-dried for 72 hrs, then finely ground, sieved (1 mm sieve) and mixed thoroughly. After mixing the sample lots were quartered and sampling for analyses was carried out by the procedure described in [9]. The determination of minerals was as follows: 0.6 g of each of the samples was dry-ashed for 3 hrs at 550 °C. The ashed samples were then dissolved in 5 ml of 6N HCl and diluted to 100 ml using deionized water. All determinations of minerals were carried out using a Perkin - Elmer AAS (model 372). Total phosphorus was determined colorimetrically by the phosphovanadomolybdate method [10].

Extraction and precipitation procedures of phytate were done by the method of Young [11]. 4g of finely ground sample was soaked in 100 ml of 2% HCl for 3hrs and then filtered through two layers of hardened filter paper. 25 ml of the filtrate was placed in a 400 ml beaker and 5 ml of 0.3% NH_4SCN solution was added as an indicator. 53.5 ml of distilled water was then added to reach the proper acidity. This mixture was titrated with ferric chloride solution, which contains about 0.00195 g of Fe per ml of $FeCl_3$ solution. Equivalent to this, the amount of phytate phosphorus was found by multiplying with a factor of 1.95. This product is again multiplied by a factor of 3.55 to convert the result into phytate.

RESULTS AND DISCUSSION

The mineral composition of the vegetables is shown in Table 2. Ca, Na, K, Mg and P are highly concentrated. The most abundant was potassium, which ranged between 98 and 2180 mg 100 g^{-1} dry matter (DM). These values are higher than those reported for land snails [12]. The present results are comparable to those reported for pigeon pea [13], agricultural grains [14) and oil seeds [15], but lower than those reported for some selected chillies [16] and mushrooms [17]. Ca1cium is the next highest mineral component, which ranged from 190-1000 mg 100 g^{-1} DM followed by magnesium, which varied from 90-460 mg 100 g^{-1} DM. It is noteworthy that calcium in conjunction with Mg, P, Mn, vitamins, Cl and proteins involves in formation of bone [15]. It also plays an important role in blood clotting, coordination of inorganic elements present in the body and balancing of Ca and P. It is very important that the normal Ca levels in the diet should be balanced throughout life.

Zinc and manganese ranged from 0.84 - 3.73 mg $100g^{-1}$ DM. These values are in agreement with those reported for corn tortillas and wheat flour tortillas [18], but lower than those reported for cowpea [19]. Cu was not detected at all. This fact should be of little concern because it is widely distributed in other types of food.

Table 3 depicts total phosphorus, phytic acid (phytate) phosphorus, phytate, phytate phosphorus expressed as percentage, and calculated phytate:Zn and Ca:phytate molar ratios. The phytate content ranged from 390-1170 mg 100 g^{-1} DM. These values are higher than those reported for other vegetables, fruits and roots [20] or mushrooms [17]. The levels of phytate are comparable to levels reported for cowpea [21, 22] and legumes [23]. Phytate contents vary considerably depending on the enviromental conditions, maturation and processing procedures [24]. Phytate chelates with mineral elements thereby have significant effects on the utilization of the minerals and also interfere with basic residues of proteins [17, 23].

The total phosphor us was highest in Egungun samples and lowest in *Amaranthus* spp. and *Vigna unguiculata*. These values are similar or in agreement with the concentrations found for soyabean, cowpea and legumes [22, 23]. Phytate phosphorus expressed as percentage of total phosphorus ranged between 40.7 - 90.9 % (only one sample shows less than 50 %).

The calculated phytate:Zn molar ratios of *Vigna unguiculata* (Cowpea) and *Solanum melongena* (Egg plant) samples are less than 20. The remaining vegetables have high phytate:Zn molar ratios. Similar ratios less than 20 have been obtained for mangoes and others and compared favourably well with cereals and legumes [20].

The phytate:Zn molar ratio of 14.8:1 could be associated with the reduced Zn bioavailability. The estimated percentage of the daily dietary energy intake supplied by maize flour in Malawi and that supplied by Tanok in Iran are comparable to these results.

Ca:phytate molar ratios of 2.9 and 26.2 are obtained in *Manihot esculenta* (Cassava leaf) and *Vigna unguiculata* samples, respectively. These results are far less to values obtained for leaves, but in agreement with values for fruits. They are above those reported for cereal flours and legumes [20]. All the vegetables` molar ratios are above the critical molar ratio of 6:l. Hence, calcium content in these vegetable diets will be sufficient to promote a phytate-induced decrease in Zn bioavailability [20].

TABLE 1
Scientific and vernacular names of the investigated vegetables.

Common Name	Vernacular Name	Scientific Name
Egg Plant	Igba	*Solanum melongena*
Bitter Cassava/(Manioc/ Tapioca leaf	Ege	*Manihot esculenta* Crantz (Euphorb)
Bitter leaf	Ewuro	*Vernonia* spp. (M Gilbert)
Green/Amaranth leaf	Tete	*Amaranthus* ssp.
Water leaf	Gbure	*Talinium triangulare*
Bush Okra	Ewedu	*Corchorus olitorius*
---	Egungun	---
Fluted gourd	Ugwu	*Telfairia occidentalis*
Okra leaf	Ila	*Abelmoschus esculentus* L. Moench (mal-
Cowpea leaf	Ewa	*Vigna uniguiculata* L. Walp

TABLE 2
Mineral concentrations in vegetable samples under investigations (mg 100 g^{-1} dry matter).

Common Name	Ca	Na	K	Mg	Zn	Cu	Fe	Mn
1. Egg plant	620	60	680	190	3.67	ND	1.00	1.34
2. Cassava leaf	190	40	170	90	1.50	ND	1.17	0.84
3. Bitter leaf	660	70	1200	200	3.34	ND	1.67	2.00
4, Green leaf	960	80	98	330	2.67	ND	0.84	2.84
5. Water leaf	1000	120	2180	460	2.00	ND	1.00	1.50
6. Bush okra	500	100	1130	210	3.67	ND	1.00	2.17
7. Egungun	700	111	1050	260	3.73	ND	0.84	2.51
8. Fluted gourd	490	90	680	100	3.67	ND	1.00	2.84
9. Okra leaf	680	50	450	140	3.67	ND	0.84	1.34
10. Cowpea leaf	620	80	530	120	2.67	ND	0.67	1.67

ND = not detected

TABLE 3
Total phosphorus (P), phytate P and phytate concentration (mg 100 g^{-1} dry matter), phytate P (% of total P), and calculated molar ratios of phytate:Zn and Ca:phytate in the vegetables examined.

Vegetable samples	Total P (mg 100 g^{-1})	Phytate P (mg 100 g^{-1})	Phytate (mg 100 g^{-1})	Phytate P (% of total P)	Phytate:Zn ratio	Ca:Phytate ratio
1. Egg plant	290	190	670	65.52	17.0	15.2
2. Cassava plant	330	300	1070	90.91	81.0	2.9
3. Bitter leaf	400	330	1170	82.50	35.4	9.3
4. Green leaf	270	240	850	88.88	32.3	18.6
5, Water leaf	290	220	780	75.86	39.3	21.1
6, Bush okra	360	270	960	75.00	24.3	8.6
7. Egungun	460	290	1030	63.04	30.4	11.5
8, Fluted gourd	440	310	1100	70.46	27.8	7.3
9. Okra leaf	310	240	850	77.42	21.5	13.2
10.Cowpea leaf	270	110	390	40.74	14.8	26.2

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AFS Book Reviews - Bücherschau

Chiral Separation Techniques – A Practical Approach

Ganapathy Subramanian (Ed.)

Second, completely revised and updated edition;
350 pages, about **200** figures and **60** tables;
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During the last two decades there was a fast-growing interest in the development and application of chiral chromatographic methods, particularly in the pharmaceutical industries but also in other branches such as agrochemical industry. The rapid development in this field is forced by the increasing pressure of regulatory authorities in the last decade against the marketing of racemic mixtures and also driven by the desire to develop and exploit "excellent science". Regulatory guidelines and progress in scientific methodologies are the power which has provided a great challenge for the industries to seek techniques that are efficient, economical and easy to apply, in the production of enantiopure products like pesticides or chiral drugs.

The versatility of chiral stationary phases and its effective application in both analytical and large-scale enantioseparation has been discussed in an earlier book "A Practical Approach to Chiral Separation by Liquid Chromatography" edited by G. Subramanian. This book aims to outline the current development and successful applications of chiral separation techiques, thereby allowing both a detailed insight to researchers and practitioners in academia and industry and a choice of methodology from the entire spectrum of available techniques. Outstanding international contributors have agreed to share their knowledge and experience with the engaged readers and have compiled a valuable source in this special important scientific field. Each chapter of this book represents an overview of its chosen topic.

FROM THE CONTENTS

Techniques in Preparative Chiral Separations by *G. Subramanian* (Introduction, Crystallization Techniques, Chromatographic Techniques, Liquid Chromatography, HPLC/MPLC, Flash Chromatography, Simulated Moving Bed, Closed-loop Recycling with Periodic Intraprofile Injection, Countercurrent Chromatography, Sub- and Supercritical Fluid Chromatography, GC, Enantioselective Membranes, Other Methods, Chiral Extractions, Preparative Gel Electrophoresis and Thin-Layer Chromatography, Enantioselective Distillations and Foam Flotation, Global Consideration, References)

Method Development and Optimization of Enantiomeric Separations Using Macrocyclic Glycopeptide Chiral Stationary Phases by *Thomas E. Beesley, J. T. Lee, Andy X. Wang* (Introduction, Characteristics of Macrocyclic Glycopeptide CSPs, Chiral Recognition Mechanisms, Multi-modal CSPs, Predictability of Enantioselectivity, Complementary Separations, Method Development with Glycopeptide CSPs, Method Development Protocols, Column Coupling Technique, Optimization, Effect of Flow Rate and Temperature on Enantiomeric Separations, Optimization of Enantiomeric Separations in the New Polar Organic Mode, Optimization of Enantiomeric Separations in Reversed Phase, Effect of Organic Modifier on Enantiomeric Separations, Effect of Aqueous Buffer on Chiral Separations, Optimization of Enantiomeric Separations in Normal Phase, Concluding Remarks, References)

Combinatorial Approaches to Recognition of Chirality: Preparation and the Use of Materials for the Separation of Enantiomers by *Frantisek Svec, Dirk Wulff, Jean M. J. Fréchet* (Introduction, Engineering of a Chiral Separation Medium, Chiral Selectors, Design of New Chiral Selectors, In Pursuit of High Selectivity, Acceleration of the Discovery Process, Reciprocal Approach, Combinatorial Chemistry, Library of Cyclic Oligopeptides as Additives to Background Electrolyte for Chiral Capillary Electrophoresis, Library of Chiral Cyclophanes, Modular Synthesis of a Mixed One-Bead - One-Selector Library, Combinatorial Libraries of Selectors for HPLC, On-Bead Solid-Phase Synthesis of Chiral Dipeptides, Reciprocal Screening of Parallel Library, Reciprocal Screening of Mixed Libraries, Library-On-Bead, Conclusion, References)

CHIRBASE: Database Current Status and Derived Research Applications Using Molecular Similarity, Decision Tree and 3D "Enantiophore" Search by *Christian Roussel, Johanna Pierrot-Sanders, Ingolf Heitmann, Patrick Piras* (Introduction, Database Status, Content and Structure, Data Registration, Searching the System, The Query Menu, The Automatic Search Too, 3D Structure Database Searches, Queries Based on CSP Receptor, Queries Based on Sample Ligand, Dealing with Molecular Similarity, Comparison of Sample Similarities within a Molecule Dataset, Comparison of Molecule Dataset Similarities between Two CSPs, Decision Tree using Application of Machine Learning, Conclusion, References)

Membranes in Chiral Separations by *M. F Kernmere, J. T. F. Keurentjes* (Introduction, Chiral Membranes, Liquid Membranes, Emulsion Liquid Membranes, Supported Liquid Membranes, Bulk Liquid Membranes, Polymer Membranes, Molecular Imprinted Polymers, Cascades of Enantioselective Membranes, Membrane-Assisted Chiral Separations, Liquid-Liquid Extraction, Liquid-Membrane Fractionation, Micellar-Enhanced Ultrafiltration, Concluding Remarks, References).

Enantiomer Separations using Designed Imprinted Chiral Phases by *Börje Sellergren* (Introduction, Molecular Imprinting Approaches, Structure-Binding Relationships, High Selectivity, Low Selectivity, Studies of the Monomer-Template Solution Structures, Adsorption Isotherms and Site Distribution, Adsorption-Desorption Kinetics and Chromatographic Band Broadening, Factors to Consider in the Synthesis of MICSPs, Factors Related to the Monomer-Template Assemblies, Influence of the Number of Template Interaction Sites, Thermodynamic Factors, Factors Related to Polymer Structure and Morphology, Methods for Combinatorial Synthesis and Screening of Large Number of MIPs, New Polymerization Techniques, Other Separation Formats, Conclusions, References)

Chiral Derivatization Chromatography by *Michael Schulte* (Introduction, Different Approaches for Derivatization Chromatography, Type 1: Covalent Derivatization with a Unichiral Derivatizing Agent, Types of Modifications for Different Groups, Separation of Amino Acid Enantiomers after Derivatization with Ortho-Phthaldialdehyde (OPA) and a Unichiral Thiol Compound, Type II: Selective Derivatization of One Compound, Type III: Increase in Selectivity, Type IV: Derivative with best Selectivity, Type V: Reactive Separation, Conclusions, References)

Nonchromatographic Solid-Phase Purification of Enantiomers by *N. E. Izatt, R. L. Bruening, K E. Krakowiak, R. M. Izatt, J. S. Bradshaw* (Introduction, Chemistry, Nonchromatographic Separation Process Description, Operating Aspects of Nonchromatograph Separation Systems, Reduced Number of Process Steps, High Chemical, Optical and Volume Yields, High-Feed Throughout, Open-Ended Solvent Choice, Minimized Solvent Usage, Low Resin Consumption, Experimental Examples of Separations, Analytical Separation of Amine Enantiomers, Automated Test Demonstration, Areas of Potential Industrial and Analytical Interest for Nonchromatographic Chiral Separations, Summary, References)

Modelling and Simulation in SMB for Chiral Purification by *Alirio E. Rodrigues, Luis S. Pais* (Introduction, The SMB Concept, Modeling of SMB Processes, The SMB Model, The TMB Model, Simulation Results, Equivalence between TMB and SMB Modeling Strategies, Separation Regions, The Steady State TMB Model, Performance Parameters, Effect of the Switch Time Intervall, Effect of the Mass Transfer Resistance on the SMB Performance, Prediction of the Separation Regions, Operation of the SMB Unit, Separation of Bi-Naphthol Enantiomers, Separation of Chiral Epoxide Enantiomers, Conclusions, References)

The Use of SMB for the Manufacture of Enantiopure Drug Suhstances: From Principle to CGMP Compliance by *S. R. Perrin, R. M. Nicoud* (Introduction, FDA as the Driving Force: (Enantiopure Drugs and Compliance), Market Exclusivity: Newly Approved Drug Substances, Fixed-Combination Dosage: Enantiopure Drug Substances, Pharmaceutical Industry: Mergers, Chromatographic Processes, SMB: Comparisons to Batch Chromatography, Illustrations of SMB Processes, SMB as a Development Tool, Basic Principles and Technical Aspects, Operating Conditions, Step A: Aquisition of Relevant Physico-Chemical Parameters, Step B: Calculation of TMB, Step C: Calculation of SMB, Example of Process Design, Manufacture of Enantiopure Drug Substances, Gathering Physico-Chemical Parameters, SMB: Linear Conditions, SMB: Nonlinear Conditions, SMB as a Production Tool, CGMP Compliance, Manufacturing and Process Controls, Solvent Recovery, In-Process Testing, Calculation of Yields and Definition of Batch, Process Validation, SMB Accepted for Manufacturing, Practical Implications for Manufacturing, Conclusions, References)

Electrophoretically-driven Preparative Chiral Separations using Cyclodextrins by *A. M. Stalcup* (Introduction, Classical Electrophoretic Chiral Separations: Batch Processes, Classical Electrophoretic Chiral Separations: Continuous Processes, Conclusions, References)

Sub- and Supercritical Fluid Chromatography for Enantiomer Separations by *Karen W. Phinney* (Introduction, Sub- and Supercritical Fluid Chromatography, Properties of Supercritical Fluids, Supercritical.Fluids as Mobile Phases, Instrumentation for SFC, Advantages of SFC for Chiral Separations, Increased Efficiency, Rapid Method Development, Column Coupling, Preparative Separations, Chiral Stationary Phases in SFC, Brush-type, Cyclodextrins, Derivatized Polysaccharides, Macrocyclic Antibiotics, Other CSPs, Method Development in Chiral SFC, Stationary Phase Selection, Modifiers, Temperature, Pressure, Flow Rate, Conclusions, References)

International Regulation of Chiral Drugs by *Sarah K. Branch* (Introduction, Requirements in the European Union, Introduction, Note for Guidance on Investigation of Chiral Active Substances, Chemistry and Pharmacy Aspects, Synthesis of the Active Substance, Quality of the Active Substance, Preclinical and Clinical Studies, Single Enantiomer, Racemate, New Single Enantiomer from Approved Racemate or New Racemate from Approved Single Enantiomer, Nonracemic Mixture from Approved Racemate or Single Enantiomer, Abridged Applications, Requirements in the United States, Introduction, Policy Statement for the Development of New Stereoisomeric Drugs, Chemistry, manufacturing and controls, Methods and Specifications, Stability, Impurity Limits, Pharmacology/Toxicology, Developing a Single Enantiomer after a Racemate is Studied, Clinical and Biopharmaceutical, Other Relevant FDA Guidance, Requirements in Japan, Guidelines from the International Conference on Harmonization, Introduction, Specifications and Tests, Impurities, Analytical Validation, Common Technical Document, The Effect of Regulatory Guidelines, Concluding Remarks, References) - **Index**

The Biotechnology of Ethanol – Classical and Future Applications

M. Roehr (Ed.)

contributing authors:
N. Kosaric, F. Vardar-Sukan, H. J. Pieper, T. Senn

232 pages, **53** tables, **53** figures;
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Progress of our civilization with an explosive rise in world population has led to an enormously increased consumption of natural resources and to an equal threat to the environment. Biotechnology is considered to play a significant role to solve these afore-mentioned problems in this century. One of the numerous examples discussed as possible alternatives and thoroughly investigated in the last few decades is the production of ethanol from various feedstocks.

Therefore, the editor in collaboration with experts in this field presents a concise overview on the state-of-the-art of manufacturing this valuable alcohol which can be used in various fields of application. Biotechnologists as well as all the people engaged in developing alternative ways of the sustainable use of renewable resources will find valuable information and useful examples described under practical aspects.

In Part I modern distillery technology using starch-containing feedstocks is covered comprehensively. Part II contains especially the usage of unconventional raw materials for ethanol production. Special attention is directed to the case of motor fuel additions and the respective implications. The data presented will help the interested readers to examine, to decide and find under a given set of conditions a suitable and economic production procedure.

FROM THE CONTENTS

Enzymes in Lipid Modification

Uwe T. Bornscheuer (Ed.)

in collaboration with:
German Society for Fat Science DFG

424 pages, numerous figures and tables**;**
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The plethora of information available down to molecular "genomic" levels still continues to grow and demonstrates that our industrial-age society is being transformed into an information-age society. Professional organizations have responded accordingly, for example the DFG created a Division of Biochemistry and Biotechnology which for some time has been co-chaired by the editor of this book, Uwe Bornscheuer. He is an outstanding expert in the field of enzyme technology. The present book "Enzymes in Lipid Modification" provides the reader with the state-of-the-art of lipid modification by lipases, phospholipases, lipoxygenases, and P450-monooxygenases. The chapters in this handbook are written by leading experts and review the latest understanding of enzyme mechanisms, recent developments in molecular biology, and biochemical engineering tools to optimize biocatalyst performance. This interesting and employable compilation opens up new areas of application for more selective modifications of fats and oils which have been traditionally used for nutrition. Modified fats and oils are recognized as valuable renewable resources for organic syntheses, flavor and fragrances, and detergents or emulsifiers. The book is a necessary supplementation in the library of biotechnologists, process engineers, food technologists, biologists and chemists.

FROM THE CONTENTS

rated Fatty Acids with Lipase by *Yuji Shimada, Akio Sugihara* and *Yoshio Tominaga;* Lipase-Catalyzed Synthesis of Structured Triacylglycerols Containing Polyunsaturated Fatty Acids - Monitoring of the Reaction and Increasing the Yield by *Tsuneo Yamane;* Enrichment of Lipids with EPA and DHA by Lipase by *Gudmundur G. Haraldsson;* Modification of Oils and Fats by Lipase-Catalyzed Interesterification: Aspects of Process Engineering by *Xuebing Xu*) **Phospholipases** (Phospholipases Used in Lipid Transformations by *Renate Ulbrich-Hofmann;* Preparation and Application of Immobilized Phospholipases by *Peter Grunwald;* Enzymatic Conversions of Glycerophospholipids by *Patrick Adlercreutz)* **Lipoxygenases** (Application of Lipoxygenases and Related Enzymes for the Preparation of Oxygenated Lipids by *Ivo Feussner* and *Hartmut Kuehn;* Properties and Applications of Lipoxygenases by *Gills Iacazio* and *Dominique Martini-Iacazio)* Miscellaneous Enzymes (Enzymatic Synthesis and Modification of Glycolipids by *Siegmund Lang*, *Christoph Syldatk* and *Udo Rau*; Fatty Acid Hydroxylations Using P450 Monooxygenases by *Ulrich Schwaneberg* and *Uwe T. Bornscheuer*); **Index**

Carbohydrates in Chemistry and Biology Vol. 1 - 4

B. Ernst, G. W. Hart, P. Sinaÿ

(Part I: Chemistry of Saccharides Vol. 1 Chemical Synthesis of Glycosides and Glycomimetics - Vol. 2 Enzymatic Synthesis of Glycosides and Carbohydrate-Receptor Interaction; Part II: Biology of Saccharides Vol. 3 Biosynthesis and Degradation of Glycoconjugates – Vol 4 Lectins and Saccharide Biology)

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It is well-known that carbohydrates play an important role in a myriad of biological systems and organisms. Glycoscience is presently among the most rapidly growing and exciting fields of chemistry and biochemistry. Due to the complexities of carbohydrate chemistry and structure, the understanding of the structural/functional relationships of glycoconjugates has lagged behind research on proteins or nucleic acids. In the past few years major advances in synthetic and analytical chemistry, molecular biology and genetics, and organism biology have put forth an enormous growth of the important role of carbohydrates. Therefore, it is now clear that virtually every major disease of infesting mankind (for example infectious diseases to cancer, cardiovascular disorders, immune disfunctions) directly involves glycoconjugates.

The editors and the collaborating authors can take credit for this outstanding comprehensive handbook consisting of short readable overviews of the state-of-the-art in the specific topics which are supplemented with key references as a gateway to more in-depth study. This essential collected edition is a valuable resource tool for both the expert in glycoscience and for the large number of researchers in other chemical, biological and biomedical disciplines where a knowledge of glycoconjugates is required.

Indeed, *Carbohydrates in Chemistry and Biology* is the most comprehensive and up-to-date compilation of information in the fields of glycochemistry and glycobiology available today. It is a must for established investigators and also advanced students in many disciplines.

FROM THE CONTENTS

Part I. Chemistry of Saccharides – Volume 1: Synthesis of Oligosaccharides (Glycoconjugates and Glycomimetics; Chemical Methods for Glycosylations: Trichloroacetimi date/Epoxides/Thiolglycosides/Phosphites/ Pentenyl Glycosides Diazirines/Alkylation of Reducing Sugars / Other; ß-Mannosides / Sialidation / Orthogonal Strategy in Oligosaccharide Synthesis/Intramolecular Glycosylations Total Synthesis and Future Aspects;

Synthesis of Oligosaccharide Mimics: C-Analogues, S-Analogues, Glycopeptoids, Combinatorial Carbohydrate Chemistry; *Enzymatic Methods for Glycosylations:* General Aspects/ Glycosyl Transferases/ Recycling of Sugar Nucleotides in Enzymatic Glycosylation/Enzymatic Glycosylation with non-natural Donors and Accetors/ Transglycosylations

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