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Rapid Quantitative Enrichment of Carnosic Acid from Rosemary (*Rosmarinus officinalis* L.) by Isoelectric Focused Adsorptive Bubble Chromatography

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For the first time, the potent but unstable antioxidative diterpene carnosic acid could be enriched from an aqueous extract of rosemary (*Rosmarinus officinalis* L.) by isoelectric focused adsorptive bubble chromatography. Enrichment of carnosic acid in the foam was influenced by the pH value and the flow rate of the foam-forming gas. Efficiency was highest with diluted samples at pH 4. Under these conditions, the conversion of carnosic acid to carnosol was negligible. Transfer of carnosic acid to the foam from a standard solution in the presence of saponin as surfactive substance was similar to that from the aqueous rosemary extract.

KEYWORDS: Rosemary; *Rosmarinus officinalis* L.; carnosic (carnosolic) acid; adsorptive bubble separation; foam separation; foam fractionation

INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.), which has long been known as a spice and medicinal herb, is receiving increasing attention due to its antimicrobial, antiinflammatory, and antioxidative constituents (*1*−*5*). The most effective antioxidant in rosemary is the diterpene carnosic (carnosolic) acid (**1**); some other, less effective diterpenes in this plant are degradation products of carnosic acid (**1**), which is mostly converted to carnosol (**2**), carnosic acid methyl ester (**3**), epirosmanol (**5**), rosmanol (**6**), and 7-methylrosmanol (**7**) (*4*, *6*−*9*) (**Figure 1**). Carnosic acid (**1**) is labile at higher pH values but stable in acid medium up to 110 °C (*10*). Temperature-dependent degradation of rosemary extracts seems to be at least partly enzymatic, because it is more pronounced in crude extracts than in standard solutions. Nevertheless, even aqueous standard solutions stored in the dark at −18 °C show a slight decrease in concentration over time, probably because of the neutral pH.

Carnosic acid (**1**) is normally obtained from methanolic extracts of rosemary. Aqueous extraction is less efficient. The disadvantage of the former method, besides the necessity to remove the toxic solvent, is the high content of fats and chlorophyll, which makes a cleanup step necessary prior to chromatographic separation of the antioxidants. To avoid these problems, the isolation and enrichment of **1** from aqueous extracts is a desirable alternative. Water as a solvent is nontoxic and cheaper than methanol; aqueous extracts are free from fats and chlorophyll, and the coextracted proteins and starch can be easily removed by precipitation. The most promising method for enrichment of this group of antioxidants is isoelectric focused adsorptive bubble chromatography (IFABC), because it is simple and inexpensive, environmentally friendly, and very effective for many mixtures containing surfactive components, such as proteins (*11*−*14*). Rosemary extracts have a sufficiently high protein content of ca. 5% (*15*) to enable an effective foam separation of nonpolar or medium-polar components. Therefore, the foaming capacity of aqueous extracts of rosemary, the possibility of enrichment of **1** from these extracts, and the stability of **1** under these conditions were investigated. The aim was to obtain the maximum yield of **1** from aqueous extracts with minimum loss by transformation.

MATERIALS AND METHODS

Chemicals. Pure crystalline carnosic acid (**1**) was kindly provided by Dr. K. Schwarz and Prof. Dr. Ternes from the University of Hannover, Germany. For the standard solution, 2 mg of this pure **1** was dissolved in 10 mL of acidic methanol (0.3 mL of H_3PO_4 in 1 L of methanol). This was diluted by the factor 50 for HPLC-ELCD measurements. Rosmaric (rosmarinic) acid (**4**) was purchased from Roth, Germany. All other compounds (**2**, **3**, **5**, **6**, and **7**) were isolated and their structures identified in a previous work (*16*) by ^1H and ^{13}C NMR. H_3PO_4 (orthophosphoric acid, 85%, p.a.) was obtained from Riedel-de Haen (Germany), and hexane (for HPLC), methanol (for HPLC), ethanol (p.a.), and NaOH (p.a.) were from Merck (Darmstadt, Germany). Tetraethylammonium hydroxide (TEAH, 20% w/w aqueous solution) was purchased from Sigma (Deisenhofen, Germany) and citric acid (puriss. p. a.) from Fluka (Germany). A mixture of pectinase and hemicellulase, for cell wall degradation (Rohapect), and saponin were obtained from Röhm (Germany).

Plant Material. Rosemary (dry and pulverized, content of **1** ca. 1.5%) was provided by Fa. Raps (Kulmbach, Germany).

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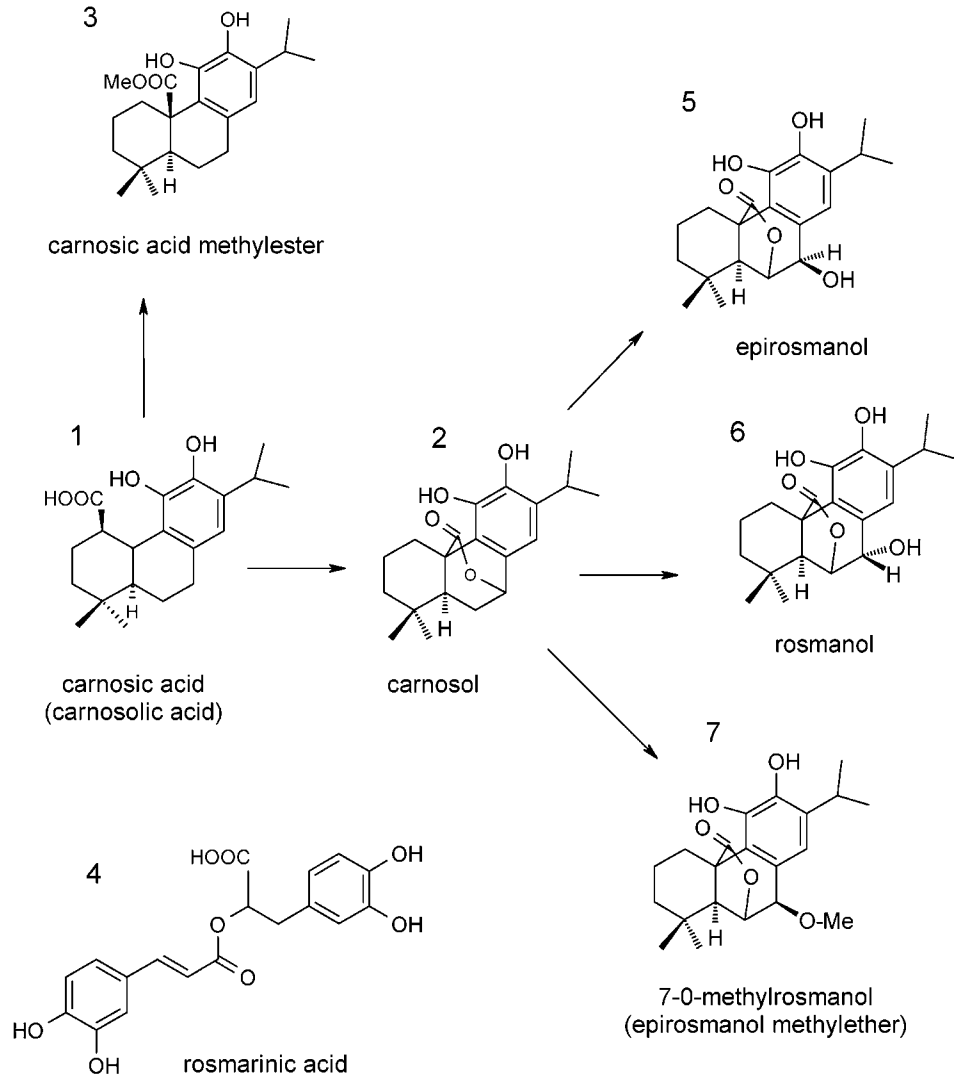

Figure 1. Structures of carnosic acid (**1**), some of its degradation products (**2**, **3**, and **5**–**7**), and rosmari(ni)c acid (**4**).

Extraction. *Methanolic Extraction.* A fixed amount of the dry rosemary (0.8 g) was extracted for 30 min with 100 mL of acidic methanol (pH 1) in an ultrasonic bath and filtered. The extract was, if necessary, filtered a second time with a membrane filter (0.45 μm) and diluted with the same solvent. The samples were then analyzed by HPLC-ELCD. The yield of **1** obtained with acidic methanol was taken as 100% (12 mg).

Aqueous Extraction. A 0.8-g amount of dry rosemary was generally heated in neutral, acidic, or alkaline water (pH 2−11.5, adjusted with 1 and 0.1 M H_3PO_4 or 0.1 or 0.25 M NaOH) to 100 °C before stirring for 20 min in an ultrasonic bath. After cooling, the extract was filtered, again adjusted to pH values from 2 to 11.5, and analyzed by HPLC-ELCD for control of yield and stability of **1** (**Table 2**, below). The filter cake was extracted with 100 mL of acidic methanol (pH 1) as described above for recovery determinations. For extraction together with cell wall degradation, 0.2 g of Rohapect was added to 0.8 g of dry rosemary in 100 mL of water, the pH was adjusted to 4.0 with 1 M H_3PO_4, and the mixture heated to 38 °C under stirring for 4 h and then filtered. To block the enzyme activities, 0.8 mg of dry rosemary was treated with 50 mL of boiling water at pH 4 for 20 min. After cooling, the 50-mL aqueous solution was extracted for 20 min under reflux.

Isoelectric Focused Adsorptive Bubble Chromatography (IFABC). The equipment consisted of a glass column (i.d. 18.5 mm, length 130 cm) with a porous frit (P3, porosity 16−40 μm) (**Figure 2**). Care was taken for the column to be extremely clean and the ground-glass free from fat. Primary experiments were done with 100 mL of standard solution of **1** (70 mg/L) and N_2, with a flow rate of 12−30 mL·min^{-1}, as carrier gas. The influence of the pH and flow rate on the antioxidant enrichment was tested to ensure a minimum loss of **1**. At fixed intervals, eluting fractions were collected and, after foam destruction by a mechanical foam breaker, used as liquid samples (spumat) directly for analysis. A 100-mL portion of the aqueous extract containing 7 mg of **1** was also separated by IFABC and analyzed in the same way as the standard solution. For identification and quantification of the antioxidants, mainly **1**, **2**, and **3**, starting solutions, and samples of spumat were analyzed by HPLC-DAD (for routine analyses), HPLC-ELCD (for potentially antioxidative components), and HPLC-MS/MS (for product identification and quantification).

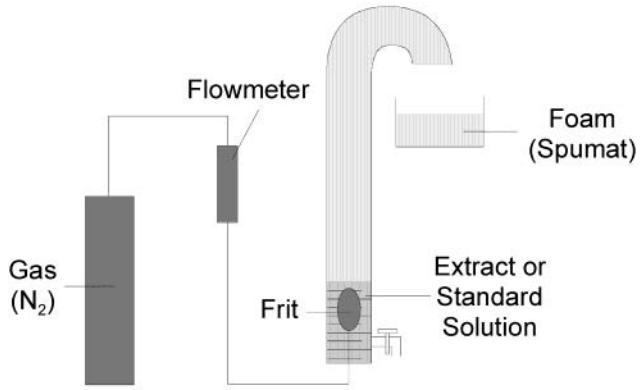

Figure 2. Equipment for isoelectric focused adsorptive bubble chromatography (IFABC).

Table 1. Time Program for HPLC-ELCD[a] Gradient Elution

time	eluent A	eluent B
0	40	60
1–6	35	65
9	20	80
10	10	90
13	0	100
14	5	95
15	10	90
16–23	40	60

[a] ELCD, electrochemical detector.

Table 2. Yield of Carsonic Acid (**1**) after Different Extractions of Dry Rosemary[a]

solvent	pH	content in filtrate (mg)[b]	content in filter cake (mg)[c]	recovery (%)[d]
acidic methanol	1	12.00 ± 0.5	0.00	100.00
water/methanol 4:1	6	7.03 ± 0.3	4.03 ± 0.2	92.17
water	2	0.30 ± 0.1	11.05 ± 0.5	92.11
water	3	1.20 ± 0.2	9.57 ± 0.5	89.75
water	4	7.00 ± 0.3	3.55 ± 0.3	88.00
water + 0.2 g of Rohapect[e]	4	7.37 ± 0.4	3.65 ± 0.3	91.83
water + hot water[e]	4	7.50 ± 0.5	3.15 ± 0.2	88.33
water	5	7.03 ± 0.4	3.07 ± 0.2	84.17
water	6	6.90 ± 0.3	2.80 ± 0.2	80.83
water	7	6.50 ± 0.3	2.73 ± 0.3	76.91
water	8	4.97 ± 0.2	2.40 ± 0.3	61.42
water	9	4.85 ± 0.3	2.08 ± 0.2	57.75
water	10	2.90 ± 0.2	1.95 ± 0.1	39.58
water	10.5	2.90 ± 0.2	1.85 ± 0.1	40.05
water	11.0	2.07 ± 0.2	1.55 ± 0.1	30.25
water	11.5	2.00 ± 0.2	1.29 ± 0.1	27.42

[a] 0.8 g of dry rosemary extracted with 100 mL of solvent under reflux for 20 min, acidic methanol extraction (see experimental part). [b] Determined with HPLC-ELCD. [c] Determined after acidic methanol extraction (100 mL) with HPLC-ELCD. [d] Calculated from contents in filtrate and filter cake. [e] See experimental part.

Isolation of Carnosic Acid (1). One liter of aqueous rosemary extract containing 70 mg of **1** was subjected to foam fractionation as described above, but with a longer column (i.d. 45 mm, length 165 cm) and a flow rate of 100 mL·min^{-1}. After foam destruction, methanol was added to the spumat to precipitate the protein fraction. The solution was filtered and cleaned by chromatography on an RP-18 column with methanol/water as eluent. The eluate was monitored by HPLC-ELCD. The elution sequence of the antioxidants was **2** (λ_{max} = 284 nm), **1** (λ_{max} = 228, 284 nm), and **3** (λ_{max} = 228, 282 nm). The latter two substances partly coeluted but could be distinguished by HPLC-DAD analysis. Fractions with pure **1** were collected, dried by rotary evaporation, dissolved in methanol, and analyzed quantitatively with HPLC-ELCD (yield, 60 mg = 85.7%).

HPLC-DAD Parameters. A Gynkotek 480 instrument with a Rheodyne 8125 injector with 20-μL sample loop, a Gynkotek UV detector (UVD 340 S, DAD; channel 1, 230 nm; channel 2, 280 nm; 3D, 210−390 nm), a Kromasil 100 C18 column (Knauer, Germany; 5 μm, 250 × 4.6 mm; column temperature, 25 °C), and a Uniflows degasser DG-1310 was used. The following gradient elution program was used: acetonitrile (eluent A) and 10 mM acetic acid (eluent B), B being 30% (0 min) → 30% (8 min) → 0% (13 min). The flow rate was 1 mL·min^{-1}.

HPLC-ELCD Analysis. A Gynkotek 480 instrument with a Rheodyne 8125 injector with 20-μL sample loop, an electrochemical detector (Antec Decade; range, 50 nA; working potential, +0.8 V; working electrode, glassy carbon; reference electrode, Ag/AgCl filled with saturated LiCl solution in MeOH/H_2O 1:1), and a Kromasil 100 C18 column (5 μm, 250 × 4.6 mm; column temperature, 25 °C) was used. Eluents for gradient elution were methanol/H_2O/2 M citric acid/TEAH 50:50:0.5:0.5 (A) and methanol/2 M citric acid/TEAH 100:1.0:0.2 (B). The flow rate was 0.8 mL·min^{-1} (**Table 1**).

HPLC-MS/MS Analysis. The same HPLC system was used as described above, but with a Gina 50 injector (injection volume, 5 μL), an MN C18 Nucleosil precolumn, an MN C18 Nucleosil 125- × 2-mm main column (column temperature, 30 °C), and the following gradient elution program: water + 1 mL of HCOOH/L (eluent A) and acetonitrile (eluent B), B being 10% (1 min) → (2.75%/min) 90 min → 90% (5 min). The flow rate was 0.25 mL·min^{-1}. The HPLC system was coupled with a Finnigan TSQ 7000 MS (vaporizer, 400 °C; capillary, 200 °C; ion source, APCI, 5 μA; sheath gas, N_2, 60 psi; auxiliary gas, N_2, 5 psi; mass range, *m*/*z* 150−500; scan time, 0.5 s).

RESULTS AND DISCUSSION

Extraction of Rosemary. First, extractions of rosemary under different conditions were compared to determine the starting amounts of antioxidants in the extracts and test the stability of **1** under the conditions used for foam separation. The yields of aqueous extraction (20 min) of dry rosemary are given in **Table 2**. Optimum values were obtained with rather low amounts (0.8 g per 100 mL of solvent) of initial material; higher ratios of dry material to solvent volume resulted in lower extraction yields. Carnosic acid (**1**) was most soluble in alkaline medium but most stable in acidic solution. The maximum yield was obtained at pH 4 with 75 mg, or 62.5% of that obtained with methanolic extraction (12 mg). Extraction with mixtures of water and methanol (4:1) gave nearly the same yields of **1** as that obtained with pure water (at pH 4) (**Table 2**). The enhancement of the extraction yield by cell wall degradation by adding Rohapect during extraction was not successful. The yield obtained was similar to that obtained with water extraction at pH 4. When hot water (near boiling temperature) was added to

Table 3. Time-Dependent Isoelectric Adsorptive Bubble Chromatography (IFABC) of Carnosic Acid (**1**) from Aqueous Rosemary Extracts[a]

	pH								
time (min)	2	3	4	5	6	7	8	9	Σ
2	0.5 ± 0.1	0.4 ± 0.1	0.2 ± 0.05	0.2 ± 0.05	0.2 ± 0.06	0.2 ± 0.06	0.2 ± 0.05	0.1 ± 0.03	2.0
3	2.4 ± 0.3	2.0 ± 0.4	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.05	0.2 ± 0.04	6.2
4	2.8 ± 0.4	2.2 ± 0.5	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3[b]	0.3[b]	0.2[b]	7.0
5	2.5 ± 0.4	2.0 ± 0.4	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.2[b]	0.2[b]	0.2[b]	6.3
6	2.3 ± 0.6	2.3 ± 0.6	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3[b]	0.2[b]	0.2[b]	6.3
7	2.2 ± 0.4	2.2 ± 0.6	0.4 ± 0.1	0.4 ± 0.1	0.4[b]	0.4[b]	0.3[b]	0.1[b]	6.0
8	2.0 ± 0.3	2.0 ± 0.4	0.4[b]	0.3[b]	0.3[b]	0.2[b]	0.2[b]	0.1[b]	5.5
9	1.5 ± 0.2	1.5 ± 0.3	0.3[b]	0.2[b]	0.1[b]	0.1[b]	0.0	0.0	3.7
10	0.5 ± 0.1	0.5 ± 0.1	0.2[b]	0.2*[b]	0.1[b]	0.0[b]	0.0	0.0	1.5
11	0.5 ± 0.1	0.2[b]	0.1[b]	0.0	0.0	0.0	0.0	0.0	0.8

[a] Absolute amount of carnosic acid (**1**) in milligrams in the different foam fractions (2–9 min; foam volume 20 mL each). Initial concentration of carnosic acid (**1**), 7 mg/100 mL; flow rate of N_2, 15 mL/min. [b] Mean values of two measurements.

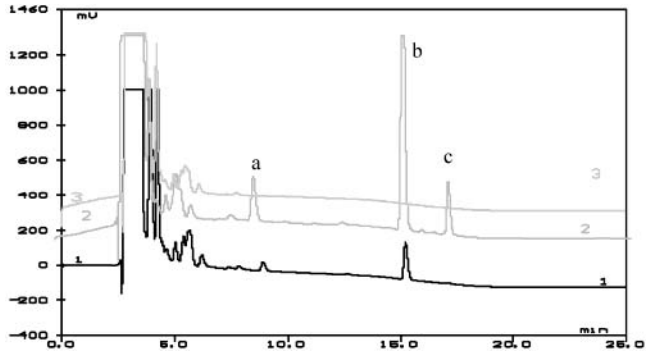

Figure 3. HPLC-ELCD chromatogram of the antioxidants of rosemary by IFABC at pH ca. 4 after 10 min. 1, extract; 2, spumat; 3, residue; a, carnosol (**2**); b, carnosic acid (**1**); c, carnosic acid methyl ester (**3**).

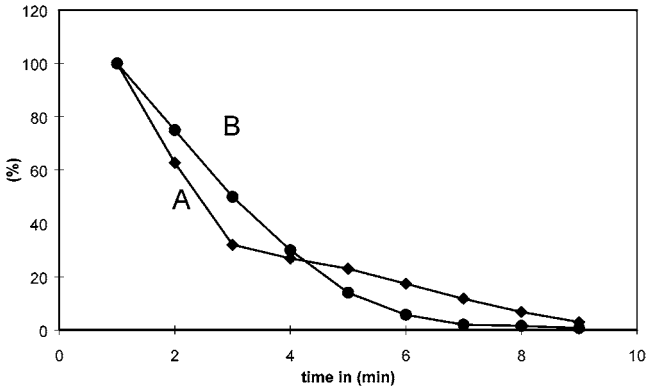

Figure 4. Decrease of carnosic acid concentration (initial 7 mg/100 mL) during foam separation of a rosemary extract (A) and a standard solution of carnosic acid (B) at pH 4.

the dry rosemary before aqueous extraction, the losses were not significantly lower, which points to negligible enzymatic degradation (**Table 2**). At higher pH values, the contact of **1** with filtrate was significantly low. Even at pH 7, the degradation of **1** under extraction conditions was remarkable. At pH 11, only 17.0 and 12.9% of the initial concentration of **1** could be detected in filtrate and filter cake.

In the filtrate, carnosol (**2**) was identified as the main conversion product. Other degradation products could not be characterized because of their extremely low levels in the extracts.

IFABC Experiments. Aqueous rosemary extract gave ample foam without addition of a surface-active substance. Addition of methanol or ethanol to the aqueous extract decreased the foam volume, and no foam was formed when the alcohol content reached 30%. This was probably due not only to the reduction of surface tension but also to protein precipitation by the organic solvent, which could be seen from the slight clouding of the solution after addition of the alcohol.

The flow rate of the gas showed only an indirect influence on the enrichment of **1** by determining the amount of foam and its water content. With a flow rate of 12 mL·min^{-1}, not enough foam was formed, and the time necessary for complete transference of **1** to the spumat had to be prolonged, while a flow rate of 30 mL·min^{-1} resulted in a foam too wet, with a high percentage of entrained extract and a low enrichment factor. A flow rate of 15 mL·min^{-1} was found to give optimal foaming conditions and transference time under our experimental conditions.

During all the experiments, a more or less enrichment of **1** into the foam could be observed (**Table 3**). No influence of the pH on foam formation was found, but the conversion of **1** to **2** showed the same dependence on the pH as that found during the aqueous extraction. Varying pH values influenced the enriching operations, and a maximum yield of 7 mg (100%) in the collapsed foam was achieved at pH 4 after 9 min of foaming. Additionally, the conversion of **1** to **2** was nearly negligible. As expected from the extraction experiments, the lowest enrichment values were obtained at pH < 2 and pH > 8, because of the rapid degradation of **1** under these conditions. At pH 2, only 2.0 mg (28.6%) of **1** could be transferred into the foam fraction after 9 min. At pH 10 and pH 11, the yields of **1** were 21.4% (1.5 mg) and 11.4% (0.8 mg), respectively.

The analysis of spumat samples taken at 1-min intervals during foam separation at pH 4 showed an effective transference of **1** to the spumat (**Figure 3**; **Table 3**). The greatest amount of **1** was transferred in a rather short time (>60% during the first 3 min), but for complete separation of the antioxidant, the foaming time had to be prolonged to 9 min. **Figure 4** shows the time dependence of transference of **1** to the spumat obtained with a standard solution using saponin as surfactive component and an extract, both at pH 4. Under these conditions, no significant differences in the extract and standard starting solution could be found.

In conclusion, these results illustrate that it is possible to obtain carnosic acid (**1**)-enriched solutions from rosemary by aqueous extraction combined with adsorptive bubble separation at pH 4 without adding a surfactant. Furthermore, **1** standard can also be transferred to the foam in the presence of a surfactant such as saponin. With both methods—conventional aqueous extraction followed by isoelectric focused adsorptive bubble chromatography—**1** can be obtained with a relatively high yield, but the absolute yields are higher with methanolic extraction. The aqueous extracts have no fats and chlorophyll and lower contents of flavors than reported in a previous work (*16*), which have to be eliminated before many applications of **1** in the food industry. A disadvantage of IFABC is the loss of part of the equally interesting antioxidative and antibacterial active rosmarinic acid (**4**), which could not be enriched in the spumat under the conditions of this study. After adsorptive bubble fractionation, **4** was always found in the same percentage in foam and starting solution. The transferability of IFABC to other complex plant extracts will be shown in further investigations.

LITERATURE CITED

(1) Brieskorn, C. H.; Dömling, J. H. Carnosolsäure, der wichtige antioxidative Inhaltsstoff des Rosmarin- und Salbeiblattes. *Z. Lebensm. Unters. Forsch.* **1969**, *141*, 10.

(2) Okamura, N.; Haraguchi, H.; Hashimoto, K.; Yagi, A. Flavonoids in *Rosmarinus officinalis* Leaves. *Phytochemistry* **1994**, *37*, 1463−1466.

(3) Reichheimer, S. L.; Bernart, M. W.; King, G. A.; Kent, M. C.; Bailey, D. T. Antioxidant activity of lipid-soluble phenolic diterpenes from rosemary. *J. Am. Oil Chem. Soc.* **1996**, *73*, 507−514.

(4) Cuvelier, M. E.; Richard, H.; Berset, C. Antioxidative Activity and Phenolic Composition of Pilot-plant and Commercial Extracts of Sage and Rosemary. *J. Am. Oil Chem. Soc.* **1996**, *73*, 645−652.

(5) Takenaka, M.; Watanabe, T.; Sugahara, K.; Harada, Y.; Yoshida, S.; Sugawara, F. New antimicrobial substances against *Streptomyces scabies* from Rosemary. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 1440−1444.

(6) Brieskorn, C. H.; Fuchs, A.; Bredenberg, J. B-son; McChesney, J. D.; Wenkert, E. The structure of carnosol. *J. Org. Chem.* **1964**, *29*, 2293−2298.

(7) Okamura, N.; Fujimoto, Y.; Kuwabara, S.; Yagi, A. High-performance liquid chromatographic determination of carnosic acid and carnosol in *Rosmarinus officinalis* and *Salvia officinalis*. *J. Chromatogr.* **1994**, *679*, 381−386.
(8) Schwarz, K.; Ternes, W. Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis* I. *Z. Lebensm. Unters. Forsch.* **1992**, *195*, 95−98.
(9) Schwarz, K.; Ternes, W. Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis* II. *Z. Lebensm. Unters. Forsch.* **1992**, *195*, 99−103.
(10) Schwarz, K.; Ternes, W. Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis* III. *Z. Lebensm. Unters. Forsch.* **1992**, *195*, 104−107.
(11) Maas, K. Adsorptive Bubble Separation Methods. In *Methodicum Chimicum*; Korte, F., Ed.; Academic Press: New York, 1974; Vol. 1A, pp 165−171.
(12) Uraizee, F.; Narsimhan, G. Foam fractionation of proteins and enzymes II. Performance and modelling. *Enzyme Microb. Technol.* **1990**, *12*, 315−316.
(13) Uraizee, F.; Narsimhan, G. Foam-based separation of proteins. In *Bioseparation Process in Foods*; Singh, R. K., Rizvi, S. S., Eds.; IFT Basic Symposium Series 10; Dekker: New York, 1995; pp 175−225.
(14) Brown, A. K.; Kaul, A.; Varley, J. Continuous foaming for protein recovery I. Recovery of β-casein. *Biotechnol. Bioeng.* **1999**, *62*, 278−290.
(15) Bundesgesundheitsamt, Germany. *Joule—Ernährungswissenschaftliches Informationssystem*; Springer: Berlin, 1993.
(16) Weinreich, B. Structure of antioxidative compounds isolated from *Rosmarinus officinalis* L. Dissertation, Technical University of Munich, Germany, 1989.

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