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Use of Reverse Micelles for the Simultaneous Extraction of Oil, Proteins, and Glucosinolates from Cruciferous Oilseeds

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Cruciferous oilseeds are important sources of oil, proteins, and glucosinolates (GLs), potentially available when biorefinery processes are used. The proposed extraction technology is based on the use of reverse micelles (RMs) made with cetyltrimethylammonium bromide (CTAB) dispersed in organic solvent. The physicochemical properties of this extraction system and the good water solubility of many high value compounds, such as GLs and some proteins, permit the simultaneous extraction of oil, and these products from cruciferous oilseed meals. This procedure is based on three main steps: (i) seed conditioning; (ii) solid–liquid extraction by RM solution; and (iii) back-transfer of the RM solution for recovery of the extracted compounds. The method makes it possible to simultaneously extract almost the same amount of oil as with pure organic solvents used in the current extraction plants and more than 90% of soluble proteins and GLs. It is a promising biorefinery technology alternative to traditional oil extraction processes.

KEYWORDS: Cruciferous oilseeds; cetyltrimethylammonium bromide; reverse micelles; simultaneous extraction; oil; glucosinolates; proteins

INTRODUCTION

Oil seed rape has been studied for several decades and is now one of the most important sources of oil and proteins worldwide. Nevertheless, less known cruciferous plants are also an interesting resource of these materials in addition to other interesting high value molecules (**Table 1**). These oilseeds cannot be exploited for food production, because of the high content of erucic acid in the oil (30–60%) and toxic and/or antinutritional compounds such as glucosinolates (GLs) in the meals (3–7%). However, at present, food is no longer the sole aim of agriculture, which is focusing more and more on molecule crops and non-food outlets. In these domains, the above-mentioned negative characteristics can turn into beneficial qualities.

High erucic acid oils (HEAOs) are suitable for a number of industrial and technical uses because of their typical fatty acid composition, rich in long chain fatty acids (more than 80%). This composition notably affects the physicochemical properties of these oils, making them very suitable not only in lipochemistry to produce erucamide, erucyl alcohol, erucate wax esters, and pelargonic and brassylic acids but also for several other uses, such as spinning lubricants in textile, steel, and metal forming industries, including rolling and drilling applications (*1–3*). For these reasons, HEAOs can substitute many petroleum derivatives, thus representing a real opportunity to decrease pollution generated by the use of fossil hydrocarbon-based products. Nevertheless, the low and discontinuous commercial availability of cruciferous seeds containing HEAO discourages the use of these oils.

Oilseed proteins are of great economic and technological significance not only for food and feed uses but also today for non-food preparations. From this point of view, the proteinic fraction of *Brassica* seeds also deserves attention because it shows interesting and positive properties besides containing a number of high value components that can be isolated using a biorefining extraction technology based on the use of the reverse micelle (RM) system as proposed in this study.

One of the most important components of medium molecular weight (MW) protein is myrosinase (MYR), which is the typical enzyme contained in all tissues of *Brassica* plants. This enzyme, because of its special physicochemical properties and its unusual stability, could be used either in soluble or in immobilized form as an industrial catalyst to hydrolyze GLs (*4, 5*). Other interesting proteins contained in *Brassica* oilseed meals are thionins and protease inhibitors (*6, 7*).

GLs are *S*-glucopyranosyl thioester secondary metabolites, which display a remarkable structural homogeneity consisting in a hydrophilic D-glucopyrano framework bearing an O-sulfated anomeric (*Z*)-thiohydroximate moiety connected to a hydrophobic aglycon side chain (*8*). Among the over 120 known GLs, the aglycon side chain may have aliphatic, arylaliphatic, or heterocyclic atom arrangements, and it is the only part of the

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Table 1. Oil, Protein, MYR, and GL Yields of Some *Brassica* Oilseeds of Potential Industrial Interest

species	oil[a] MT[e]/ha	main fatty acid (%)	proteins[a] MT/ha	MYR[b] U × 10^6/ha	GLs (kg/ha)	GLs (main type) (%)
Brassica napus (HEAO)	0.9–1.6	erucic (~45)	0.5–0.8	40–60	34–78[c]	progoitrin (~70)
Crambe abyssinica[d]	0.6–1.2	erucic (~55)	0.5–0.8	57–102	60–90	*epi*-progoitrin (~90)
Sinapis alba	0.4–0.6	erucic (~40)	0.5–0.7	50–110	100–140	glucosinalbin (~100)
Brassica juncea	0.5–0.7	erucic (~31)	0.5–0.6	6–13	70–90	sinigrin (~90)
Brassica carinata	0.6–1.2	erucic (~38)	0.6–0.9	16–24	80–150	sinigrin (~97)
Brassica nigra	0.5–0.7	erucic (~36)	0.6–0.8	8–15	120–160	sinigrin (~95)
Eruca sativa	0.5–0.9	erucic (~45)	0.4–0.7	15–25	75–140	glucoerucin (~95)
Lesquerella fendleri	0.1–0.4	lesquerolic (~70)	n.d.	n.d.	20–100	glucoiberin (~100)
Limnanthes alba	0.1–0.3	gadoleic (~60)	0.1–0.3	7–15	10–30	glucolimnanthin (~100)
Barbarea verna	0.2–0.6	erucic (~45)	0.4–0.6	3–6	100–160	gluconasturtiin (~100)

[a] Data of field experiments and literature. [b] Total MYR activity determined in seed meal samples with sinigrin as a substrate by pH stat method. [c] From Fenwick et al. (*34*). [d] Data based on the results of the DICRA project (FAIR CT 98-4333). [e] MT: metric tons.

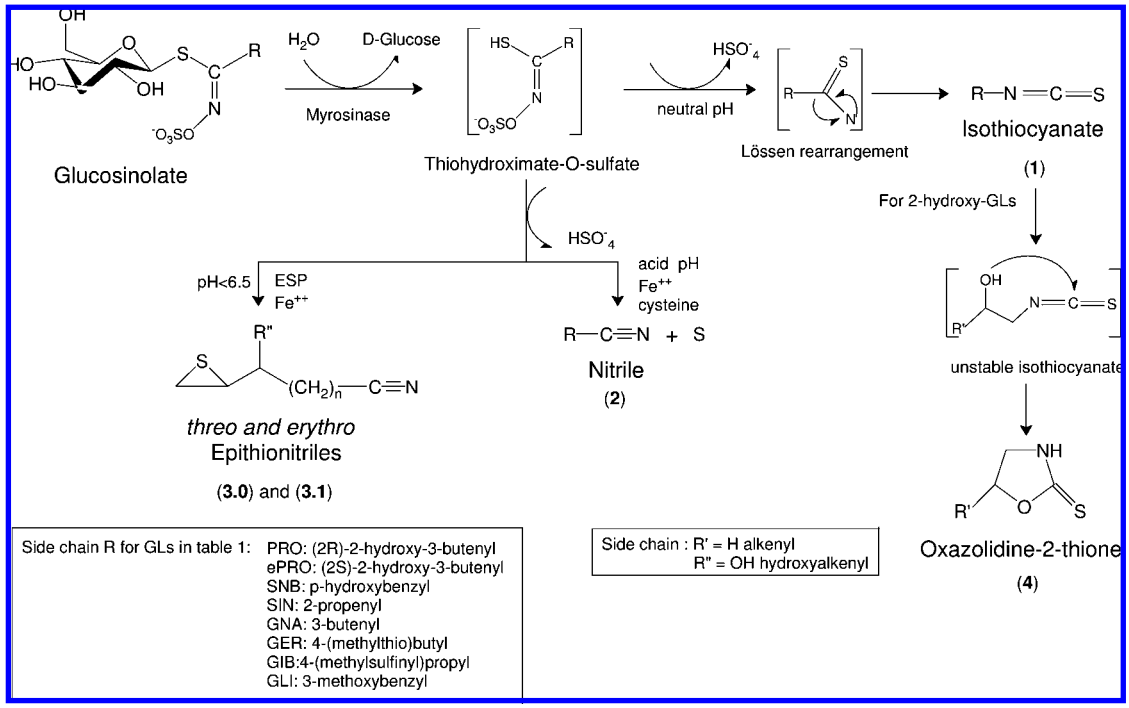


Figure 1. General pathway of myrosinase-catalyzed hydrolysis of glucosinolate.

molecule that determines the structural molecular variation of GLs. One of the most important common properties of GLs is their aptitude to produce, through myrosinase (MYR)-catalyzed hydrolysis, a number of breakdown products, mostly isothiocyanates (ITCs), long known for their fungicidal, bactericidal and nematocidal properties (**Figure 1**) (*9*). When seeds are processed with the current extraction techniques, most GLs remain in the meal making it unsuitable for animal nutrition. This is why oilseed rape has been genetically improved in order to decrease its GL content, making oilseed rape meal largely available for animal nutrition. Besides this use, an easy method to extract the GLs and other bioactive compounds would have additional advantages.

At present, GLs are not commercially offered on an industrial scale, and only a few of them are available as fine chemicals. Today, GLs can be indirectly used on a large scale as components of *Brassica* tissues and seeds only. Consequently, the cost-effective production of GL concentrated extracts appears to be an important way to make these compounds available for industrial chemistry, food technology and agriculture. The approach based on the use of RMs dispersed in organic solvent offers a new and decisive potential to this purpose.

RMs are spheroidal aggregates, with a diameter ranging from 10 to 100 Å, formed by an amphiphilic surfactant in organic solvent (*10*). The hydrophilic part of the surfactant, neutral or positively/negatively charged, is directed toward the interior of the micelle and forms a polar core in which water can be solubilized. Although the term water-in-oil microemulsion is more properly used for the surfactant/organic solvent/water ternary system, containing a larger amount of water (more than 2%), in this article we will use simply the term reverse micelles. The water content of RM solutions is expressed as Wo, the molar ratio between water and surfactant.

The good water solubility of all GLs, whatever their chemical structure, is usefully exploited for the extraction and separation of these compounds from solid state systems such as cruciferous oilseed meals by a RM system. This technology has already been successfully used to extract amino acids, peptides, and proteins not only from aqueous solutions but also from solid materials, as in the case of the simultaneous extraction of oil and proteins from sunflower and soybean meals, including GLs from different vegetable oilseed meals (*11–13*).

An extended use of a new extraction technology is a possible way to reduce the cost of several biobased products, such as HEAOs, proteins, and some bioactive compounds, making them competitive. An integrated innovative biorefinery process thus appears to be economically appropriate not only to enhance the

general value of oilseeds but also to improve the market of their derived products.

The present study describes a procedure that permits the simultaneous extraction of oil, proteins, and GLs from different cruciferous oilseeds. The results indicate that this technology has the requirements to be set up and developed on an industrial scale, representing a reliable and convenient alternative to the current extraction procedures, allowing the diversification of the oilseed extraction products without greatly modifying the current solvent extraction plants.

MATERIALS AND METHODS

Plant Material. The seeds of *B. carinata* (*Brassica carinata*) cv. ISCI 7-2005 and *B. juncea* (*Brassica juncea*) cv. ISCI 20-2005 were purchased from Cerealtoscana S.p.A, Livorno, Italy. *Crambe* (*Crambe abyssinica*) seeds, cv. Cebeco 9402, were obtained from Cebeco, Rotterdam, The Netherlands.

Materials and Reagents. Cetyltrimethylammonium bromide (CTAB) and Eosin Y were provided by Sigma-Aldrich. Polyethylene glycol *tert*-octylphenyl ether (TX-100), polyoxyethylenesorbitan trioleate (Tween 85), and bis(2-ethylhexyl)sodium sulfosuccinate (AOT) were obtained from Fluka. Nylon 6.6 net (Eterlon) filters were supplied by Gaudenzi (Padova, Italy). The other reagents were of analytical grade and were purchased from Carlo Erba, Merck or Acros organics.

Glucosinolates. The GLs used as standards were sinigrin (2-propenyl glucosinolate) and *epi*-progoitrin [(2*S*)-2-hydroxy-3-butenyl glucosinolate]. These GLs were purified according to the method proposed by Thies (*14*) with some modifications (*15*), starting from mature seeds of *B. carinata* or *B. juncea* for sinigrin isolation and *Crambe* for *epi*-progoitrin.

MYR Deactivation and Oilseed Meal Preparation. Intact seeds were placed in Pyrex bottles and warmed up at 120 °C under 1 bar of pressure in an autoclave for different times according to the type of seed considered. After thermal conditioning, the seeds were cooled at room temperature, then milled in a coffee grinder. The meal was sifted through a 0.75 mm sieve to prepare a homogeneous sample that was stored at room temperature in a sealed vial. This procedure was omitted in the case of MYR isolation.

GL Hydrolysis Product Analysis. The efficiency of the MYR deactivation procedure was tested by determining the hydrolysis products formed by this enzyme from the main GL in the meal after water addition: (5*R*)-5-vinyl-1,3-oxalidine-2-thione (VOT), from *epi*-progoitrin in *Crambe* meals and allyl isothiocyanate (AITC) from sinigrin in *B. carinata* and *B. juncea* meals. The VOT was determined according to the method reported by Leoni et al. (*16*) and analyzed by HPLC with a Hewlett-Packard chromatograph 1100 series equipped with a diode array as detector and an Inertsil 5 ODS-3 column (250 × 3.0 mm). Exploiting its volatility, the AITC was determined by headspace GC analysis, using a Carlo Erba GC-FID system, model HRGC 5300, following a procedure set up in our laboratories.

Preparation of RM Solutions. When necessary, the surfactants Triton X-100, Tween 85, CTAB, or AOT were first solubilized or suspended in a cosurfactant (like *n*-butanol or isopropyl alcohol). The latter was necessary in most cases to facilitate the formation of RM aggregates (*12, 17*). This blend was then dissolved in the required amount of organic solvent (hexane, isooctane, or cyclohexane) to form a solution or suspension at a prefixed surfactant concentration (*18, 19*). A suitable amount of water (or buffered solution) was finally added to obtain a micellar solution at the desired Wo ($[H_2O]/[surfactant]$) (*10*). The RM solution was gently shaken until a clear solution was formed.

Forward Extraction Step from Meals. Deactivated meal (1 g) (or nondeactivated in the case of MYR extraction) was mixed with 50 mL of a RM solution (1:50 w/v) by gentle magnetic stirring at room temperature for 1 h. The mixture was then left to settle to allow phase separation. The supernatant solution was clarified by filtration through four Eterlon filters (20 μm) previously washed with isooctane. The remaining meal was stored for characterization and analyses. In the case of protein and MYR extraction, the aqueous fraction of the RM solutions was 50 mM phosphate buffer at pH 7.5. In order to determine the protein and MYR soluble fraction in the meal, crude extracts were also prepared using 50 mM phosphate buffer at pH 7.5, under the conditions described above: meal and buffer solution 1:50 w/v and 1 h extraction time. For a comparison of the extraction yield achieved with RM solution and buffer, the soluble proteins extracted with the RM system were expressed as % of proteins determined in the crude extract. Oil extraction was also performed with organic solvents, such as isooctane or hexane, following the same procedure as that with RM and buffer extraction. The oil extraction yield was calculated from the oil analysis of the remaining meal after RM and organic solvent extractions. GL, protein, MYR, and oil extractions were carried out at least three times for statistical analysis.

Back-Transfer Step from RM Solutions. After forward extraction, the water soluble compounds such as GLs, proteins, and MYR, confined in the water fraction, and the oil solubilized in the organic phase of RM system were recovered in separated aqueous and organic solutions, respectively, using a back-transfer technique. Depending on the experiments, back transfer was carried out simply with water or with AOT in aqueous solution.

Back Transfer with Water. An equal volume of distilled water was mixed with the RM solution obtained from the extraction step, after meal separation. Only in the case of 50 mM CTAB, a 1:2 ratio between water and RM solution was sufficient for the back-transfer process. The mixture was stirred for 30 min at room temperature with a magnetic stirrer and then centrifuged at 30,100*g* in order to obtain a phase separation. The bottom aqueous phase was withdrawn from the centrifuge tube and analyzed for GL content. The upper organic phase contained the oil. In the case of the RM solution formed with Tween 85, water addition alone did not allow any phase separation, even by centrifugation. In order to obtain a two-phase system that permits aqueous phase recovery, an addition of an alcohol (60% v/v in water) such as isopropyl alcohol was necessary (*20*).

Back Transfer with AOT. An equimolar amount of AOT was added to the CTAB RM solution together with an equal volume of distilled water or 50 mM phosphate buffer at pH 7.5 for protein and MYR extraction. The aqueous and organic phases were recovered following the same procedure previously described for the back transfer with water. The aqueous phase obtained was then ready for protein, GL, and MYR analyses.

CTAB Detection. CTAB in water phase after the back transfer with AOT was qualitatively determined with a colorimetric assay. This assay was based on the reaction between eosin and CTAB, which forms a red precipitate as described by Furlong and Elliker (*21*). The aqueous phase (2 mL) was added to 2 mL of dichloromethane in a test tube, together with 0.5 mL of 0.1 M sodium citrate buffer at pH 4.5 and 0.2 mL of eosin yellowish dye (0.5 mg/ml water solution). A pink to red color in the dichloromethane phase indicated the presence of CTAB (up to 1 ppm).

GL Extraction by Percolation. Deactivated *Crambe* meal (1 g) was laid on a sieve over four Eterlon (20 μm) filters previously washed with isooctane. Fifty milliliters of 100 mM CTAB/isooctane/10% *n*-butanol RM solution, Wo 10, was fed through the meal layer by gravity. After this single extraction step, the RM solution was used to perform the back-transfer procedure with water to recover all of the water soluble materials. The aqueous solution was then analyzed for GL content.

Quantitative GL Analysis. GL analysis was performed following the standard method based on the HPLC analysis of desulfo derivatives with some modifications (*22, 23*), using a Hewlett-Packard chromatograph 1100 series equipped with a diode array as detector and an Inertsil 5 ODS-3 column (250 × 3.0 mm).

MYR Assay. After AOT back transfer of the RM solution, the obtained buffered aqueous extract was dialyzed against 50 mM phosphate buffer at pH 7.5 in order to eliminate or reduce any residual solvent, such as *n*-butanol, before MYR detection. The activity of soluble MYR was then determined by spectrophotometric analysis, performed with a Cary Model 219 recording spectrophotometer, equipped with a thermostatted cell compartment, as previously described (*24*). Total MYR activity in the meals was determined using the pH stat method with a Mettler Toledo DL50 titrator according to the

Table 2. Oil Content of the Meal (Soxhlet Analysis) and Percent of Extracted Oil with Isooctane and RM System (100 mM CTAB in Isooctane and 10% *n*-Butanol, Wo = 10; 1:50 w/v)

oilseed species	oil content % (w/w)	extracted oil with isooctane %[a]	extracted oil with RM system %[a]
C. abyssinica cv. Cebeco 9402-NL	50.4 ± 0.1	95.1 ± 0.7	97.8 ± 0.3
B. carinata cv. ISCI 7-2005	31.9 ± 0.4	96.3 ± 0.5	94.5 ± 1.5
B. juncea cv. ISCI 20-2005	43.5 ± 0.2	95.8 ± 0.5	95.2 ± 0.1

[a] Oil extraction yield was calculated as the difference between oil meal content before and after extraction.

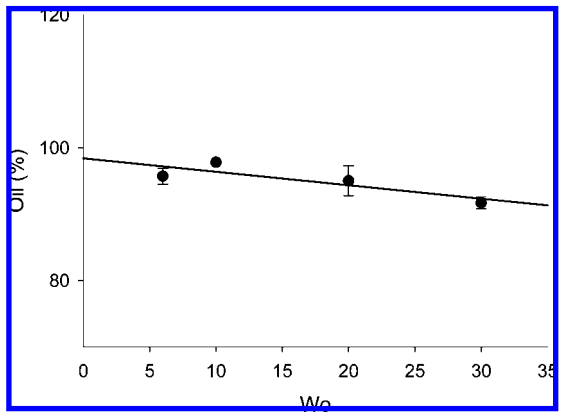


Figure 2. Oil extraction from *Crambe* meal by CTAB RM system as a function of Wo (100 mM CTAB in isooctane and 10% *n*-butanol). Wo vs percent of extracted oil in the organic phase (●—●). Oil extraction yield was calculated as the difference of oil meal content before and after RM extraction.

procedure used by Finiguerra et al. (*25*). One unit of MYR activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of substrate per minute under the assay conditions. MYR activity was also determined in the crude extract prepared using 50 mM phosphate buffer at pH 7.5 instead of the RM solution, as described above.

Oil Analysis. The oil content of the meal, before and after extraction with RM and organic solvents, was determined with the standard Soxhlet extraction method. Oil extraction yield was calculated as the difference between the initial and the final oil content of the meal.

Protein Analysis. The buffered aqueous phase, obtained after the AOT back-transfer step, and the crude extract were dialyzed against 10 mM phosphate buffer, freeze-dried, and dissolved in water in order to obtain a concentrated extract with a low ionic strength and without any residual solvent. The soluble protein concentration of these solutions was determined with the biuret method (*26*) with bovine serum albumin as standard (4–40 mg/mL). The total protein content of the meal was determined by the standard Kjeldahl nitrogen analytical procedure.

SDS−PAGE. The concentrated aqueous extracts of proteins were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) with a Phastsystem apparatus (Pharmacia LKB) using homogeneous Phastgel 12.5% and Coomassie blue staining techniques. Pharmacia standards and protocols were also used.

RESULTS AND DISCUSSION

Oil. The proposed extraction technology makes it possible to extract roughly the same amount of oil as with pure isooctane or other non polar organic solvents, whatever the seed meals used (**Table 2**). In addition, the use of different surfactants, that is, CTAB and AOT, did not affect the oil extraction significantly (data not shown). Furthermore, the oil extraction yield with CTAB RM solutions was also quite independent of Wo (**Figure 2**). On the basis of these results, it is reasonable to envisage that the new extraction process should allow as large an oil extraction capacity as with the current solvent extraction method. This result is fundamental because, in any case, the oil remains the most important constituents of the oilseeds.

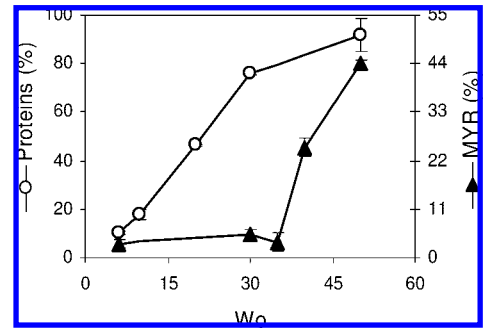


Figure 3. Soluble protein and MYR extraction yield from *B. carinata* meal by 100 mM CTAB RMs in isooctane and 10% *n*-butanol, as a function of Wo. Extracted soluble protein % (○—○); extracted soluble MYR % (▲—▲). Soluble proteins and MYR extraction yields are expressed as percent on their content of the crude extract.

Proteins. In *Brassica* seeds, the raw protein content ranges from 20% to 40%. **Figure 3** shows the percentage of soluble protein extracted from *B. carinata* meal by the RM system as a function of Wo. From low water concentrations Wo 6, up to Wo 30, the extraction yield was proportional to Wo, and from this point on, the curve levels off. Compared with soluble proteins extracted with phosphate buffer, the RM system at low Wo made it possible to extract only a small part of soluble proteins (ca. 10% at Wo 6), while at Wo 50, almost the total amount of soluble proteins was isolated (91.7%). These results were somewhat expected because a RM system with a higher water concentration increases its extraction capacity of water soluble molecules. In addition, the increased dimension of RM, associated with higher Wo (*10*), can host larger soluble proteins, which presumably prevail in the protein fraction of *Brassica* oilseeds. Electrophoresis analysis of the aqueous extracts obtained at different Wo levels, from *B. carinata*, showed the same relationship between Wo and MW (results not shown), similar to previous results reported by Leser et al. on protein RM extraction from sunflower and soybean meals (*11*). Extraction of MYR (MW ∼140 kDa) from not heat-treated *B. carinata* meal also confirmed this trend: MYR was extracted to a greater extent by RM solution with Wo 40 and 50 and was detected only in traces in extracts obtained with lower Wo (**Figure 3**). This result appears important because it shows that the RM system makes it possible to separate different kinds of proteins on the basis of their molecular mass.

Glucosinolates. With the aim of setting up an efficient extraction technique of the most important cruciferous oilseed components including GLs, several trials have been carried out using anionic, cationic and neutral surfactants, viz., AOT, CTAB, Triton X-100, and Tween 85, respectively. These extraction trials were performed using *Crambe* seed meals with the above surfactants at Wo 6 to evaluate the efficiency of GL extractability at low water concentrations with different RM systems. The results reported in **Table 3** highlight first of all the capacity of GLs to be solubilized in the water fraction of RM because of their high hydrophilic properties, which, in any case, allows a strong action in the solid–liquid phase extraction of these compounds. In particular, the CTAB RM system clearly proves to be the most efficient for GL extraction from *Crambe* seeds, thus demonstrating that the electrostatic interaction between oppositely charged CTAB and GLs is the main factor responsible for their isolation in such a high concentration,

Table 3. GL Extraction from *Crambe* Meal by RM Technique in Batch (1:50 w/v) Using Different Surfactants at Wo 6

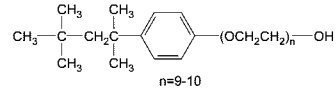
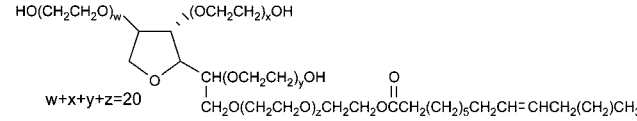
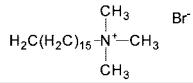
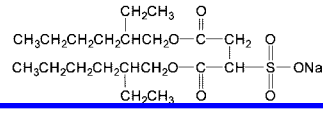
Solvents & Surfactants		Total extracted GLs (%)
Triton X-100 100 mM in cyclohexane + n-butanol 4:1 (w/v)		54.7 ± 2.8
Tween 85 100 mM in hexane + 8% isopropylalcohol (v/v)		56.5 ± 1.2
CTAB 100 mM in isooctane + 5% n-butanol alcohol (v/v)		82.9 ± 4.1
AOT 100 mM in isooctane		35.3 ± 2.7

Table 4. MYR Deactivation and RM GL Extraction from Some *Brassica* Seed Meals[a]

species	total MYR activity (U/g meal)	deactivation time of MYR at 120 °C (min)	total GLs (μmol/g meal)	GL content after MYR deactivation (μmol/g meal)	extracted GLs (%)
C. abyssinica cv. Cebeco 9402-NL	45.7 ± 2.5	17	97.2 ± 0.9	89.7 ± 0.4	86.3 ± 4.0
B. carinata cv. ISCI 7-2005	6.7 ± 0.2	5	119.0 ± 0.8	101.1 ± 1.0	83.2 ± 2.5
B. juncea cv. ISCI 20-2005	3.9 ± 0.1	5	89.8 ± 1.0	79.0 ± 1.2	83.0 ± 1.2

[a] RM technique in batch (100 mM CTAB in isooctane and 10% *n*-butanol, Wo = 10; 1:50 w/v).

contributing to keep them confined within the water fraction of RM. This finding is in agreement not only with the lower extraction yield obtained with the anionic surfactant AOT but also with the extraction yields obtained using neutral surfactants, where the driving force that allows GL extraction mostly depends on their hydrophilic properties. The efficiency of the RM extraction technology for GLs was also tested with *B. carinata* and *B. juncea*. The RM solution made up with 100 mM CTAB in isooctane and 10% *n*-butanol at Wo 10 gave almost the same GL % yields in all three species (**Table 4**). An additional factor that notably affects the efficiency of the extraction procedure is the ratio between the solid phase to be treated and the liquid extractive phase, that is, the RM system in our case. The extraction volume and the percentage of extracted GLs increased proportionally up to a ratio of 1: 25 (w/v) (data not shown); this value indicates the optimal operative proportion between the solid and liquid extractive phases.

Finally, the most important element affecting RM extraction is the water content of the system, namely, Wo. The relationship between Wo and GL extraction yield was evaluated starting from *Crambe* and *B. carinata* meals, with 100 mM CTAB, as shown in **Figure 4**. In both cases, because of their ionic nature, low MW, and high water solubility, GLs were mostly extracted at low Wo (84% at Wo 10), and from this point on, the extraction yield slightly increased up to Wo 30 (more than 90%). A different behavior was observed in the case of protein extraction at various Wo levels: the soluble protein extraction yield was in fact rather directly proportional to the Wo (**Figure 3**), as discussed above. Similar experiments were carried out with CTAB at a lower concentration (50 mM) on *Crambe* meal. **Figure 4** shows that 100 mM CTAB allowed the extraction of the highest percentage of GLs in all ranges of Wo and that the difference between the two CTAB concentrations used was modest. All data of GL extraction yield percentage were confirmed by GL analyses of the remaining meals after

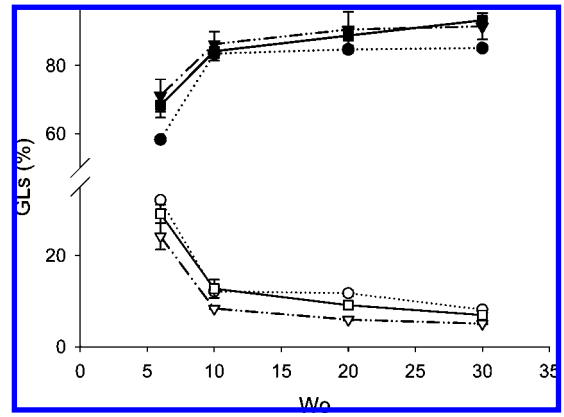


Figure 4. GL extraction yield from *Crambe* and *B. carinata* meals by CTAB RMs in isooctane and 10% *n*-butanol, as a function of Wo. Crambe with 100 mM [CTAB] (▼—••—▼) and 50 mM [CTAB] (●•••●); *B. carinata* with 100 mM [CTAB] (■—■). Residual GL content in *Crambe* meal 100 mM CTAB (▽—••—▽) and 50 mM CTAB (○•••○); residual GL content in *B. carinata* meal and 100 mM [CTAB] (□—□).

extraction. These experiments indicate that in CTAB RM systems the water content affects GL and soluble protein extractability differently, showing that Wo 10 is the critical threshold for an optimal GL extraction yield. An RM system with Wo 10 does in fact make it possible to obtain a meal with less than 10% of the original GL content, while proteins, still present in the meal, could be fractionated with subsequent extractions, in a discontinuous mode, using RM solutions at higher Wo levels (**Table 5**).

Extraction Process. The main steps of this process are (i) seed conditioning, MYR and other enzyme deactivation, in the case of GL, and protein extraction; (ii) solid–liquid phase extraction of GLs and soluble proteins from the oilmeal in the water fraction of RMs; and (iii) back transfer in which the

Table 5. Optimal Conditions for Oil, GL, and Protein Extraction to Apply RM Extraction Technology in a Discontinuous Mode, Determined on *Crambe*, *B. carinata*, and *B. juncea* Oil Meals

extraction steps	extraction conditions
(i) Seed conditioning and enzyme deactivation	5 min at 120 °C[a]
(ii) Solid–liquid extraction:	
solid–liquid ratio	1:25 (w/v)
surfactant	CTAB
[surfactant]	100 mM
cosurfactant	*n*-butanol
Wo GL extraction	10
protein extraction	15–30
time	1 h
(iii) Back transfer	
added solution	water
ratio	1:1
time	30 min

[a] 17 min at 120 °C for *Crambe*.

extracted GLs and proteins are released from RMs and recovered in aqueous solution. Although not experimented in this study, two additional steps have to be considered: (iv) surfactant removal, recovery, and reuse; and (v) oil refining. (See **Table 5** for the technical details of each step.)

Meal Conditioning and MYR Deactivation. Traces of MYR activity in the seeds catalyze the production from GL of degradation products (DP) (**Figure 1**) in the presence of water contained in the RM fraction, affecting the RM extraction efficiency. For this reason, MYR was completely deactivated before the milling procedure. Several trials carried out in an autoclave at different temperatures and times have demonstrated that a treatment of 17 min at 120 °C was sufficient for complete MYR deactivation in *Crambe* meals, whereas only 5 min at 120 °C was enough to deactivate this enzyme in *B. carinata* and *B. juncea* (**Tables 4** and **5**). This treatment also caused some GL degradation, quantifiable on average in not more than 12% of total GLs (GL meal content before and after MYR deactivation is shown in **Table 4**).

Solid–Liquid Phase Extraction. The simultaneous extraction of oil and water soluble compounds from different oilmeals occurred in a short time using RM extraction solutions. Preliminary experiments demonstrated that 1 h of extraction time was sufficient to completely extract the seed components. Oil is immediately dissolved in the organic phase, whereas the water soluble compounds, essentially GLs and proteins in crucifer oilseeds, are extracted and confined in the RM water fraction. Among the extraction steps, this one is decisive for determining the major benefit of the new procedure also in view of its use in a continuous mode. In this regard, an extraction experiment carried out by percolation of an RM solution containing 100 mM CTAB and 10% *n*-butanol with Wo 10 was performed in a single step, using the same meal/RM solutions (w/v) ratio as that usually used in this study. With this experiment, we established that almost 53% of the total GLs contained in *Crambe* meal was removed in a single step. It is reasonable to think that with continuous repeated extractions by the percolation system, as current extractors usually do, almost all of the GLs contained in *Brassica* oil seed meal should be removed.

Back Transfer and Recovery of GLs and Proteins in an Aqueous Solution. This is a very important step of the extraction procedure that follows the removal of the solid phase from the RM solution. With the aim of disrupting RMs and recovering the water soluble material such as, in our case, GLs and soluble proteins, we used high ionic strength solutions (for instance 1 M NaCl or K_2SO_4) as reported in other studies (*11, 12*). This treatment does not, however, seem so convenient at an industrial level, given the amount of inorganic salts required for this process. More functional alternative methods, with a comparable recovery yield, were therefore studied.

Back Transfer with Water. The addition of water in sufficient amounts to the RM extraction solution, that is, in the ratio of 1:1 or 1:2, has been found to be the most simple and efficient procedure, presumably also practical for large scale application. In this way, GLs and soluble proteins were recovered only in the water phase, while the oil was in the organic phase. The surfactant and cosurfactant, *n*-butanol or isopropyl alcohol, were mostly distributed in the aqueous phase.

Back Transfer with AOT. After the back-transfer process, most of the CTAB was distributed in the aqueous phase from which it had to be removed. Counterionic surfactants are well suited for this aim. For this purpose, we used AOT, and in this case, the back-transfer mechanism is presumed to be caused by the formation of a CTAB−AOT complex, inducing the RM to collapse (*27*). The hydrophobic AOT−CTAB complex was removed with organic solvents. This technique allowed a fast phase separation between the organic and water phases, without any interphase formation. An additional advantage of this procedure was that the protein analysis with current methods (such as the biuret or Bradford methods) became possible, while GL extraction yield and analyses were as easy as that with the water back-transfer method. However, it is important to mention that with this back-transfer method part of the AOT remained in the aqueous phase.

The solubilization of pure proteins, GLs, and MYR in RM solutions by liquid–liquid and solid–liquid phase extraction methods have been previously widely demonstrated by Leser and Luisi, Hochkoeppler and Palmieri, and Hayes (*12, 28, 29*). However, the ability of RM solutions to extract oil, proteins, and GLs starting from cruciferous oilseed meals has not been previously studied. Even though this extraction procedure has been set up at the laboratory level, it appears to be practical on an industrial scale in view of the small number of adjustments and implementation necessary to adapt the current oilseed extraction plants to the new procedure. The method could be susceptible to improvement not only in terms of the operating system but also with regard to safety and environmental impact. For instance, the use of RMs in supercritical CO_2 instead of hydrocarbon solvents could satisfy this requirement (*30*). The RM method has been developed with the aim of extracting and adding value to most of the oilseed components as much as possible. In principle, this procedure appears to be practical also for the extraction of other oilseed species, viz., sunflower, soybean, cottonseed, and linseed, which, instead of GLs, contain other water soluble biological active molecules that could be isolated, such as chlorogenic acid, flavonoid glucosides (genisteins), gossypol, and cyanogenic glucosides, respectively. The existing extraction techniques essentially provide only two products, the oil and the remaining proteinic part, the so-called oilcake, whereas the proposed procedure is able to simultaneously extract some of the main cruciferous oilseed components such as oil, soluble and bioactive proteins, and GLs. In addition, at the end of the extraction procedure a solid proteinic GL-free fraction is obtained, which is exploitable for some non-food applications.

In our opinion, the large availability of different kinds of GLs, at least in the form of concentrated extracts, is one way to open up the use of these compounds in agriculture, especially in crop protection as a valid alternative to methyl bromide whose use in agriculture has been forbidden in western countries since 2005. In addition, a greater number of agricultural applications are possible

using pure components or concentrated extracts containing the MYR-GL system in appropriate formulations (*31, 32*).

Finally, the past decade has witnessed an increasing interest in GLs and MYR being used not only in agriculture but also in fine chemistry and food technology (*33*). In particular, thio-functionalized GLs (glucoiberin, glucocheirolin, glucoerucin, and glucoraphanin), some alkyl GLs (sinigrin and glucocapparin), and arylalkyl GLs (glucotropaeolin and gluconasturtin), which are precursors of molecules with high biological activity, should be considered as important specialty chemicals with high economic value. These compounds, together with some antioxidants, seem to be optimal candidates for use in different forms in cancer prevention, thus notably increasing their economic value, which could support the increased cost of seed processing. For these reasons, given that the RM extraction method is particularly suitable for large scale isolation of oil and high value water soluble molecules from cruciferous oilseeds, it appears to be an economic alternative to the current extraction processes.

ABBREVIATIONS USED

GL, glucosinolate; HEAO, high erucic acid oil; RM, reverse micelle; MW, molecular weight; MYR, myrosinase; ITC, isothiocyanate; CTAB, cetyltrimethylammonium bromide; TX-100, polyethylene glycol *tert*-octylphenyl ether; Tween 85, polyoxyethylenesorbitan trioleate; AOT, bis(2-ethylhexyl)sodium sulfosuccinate; VOT, (5*R*)-5-vinyl-1,3-oxalidine-2-thione; AITC, allyl isothiocyanate; GL DP, glucosinolate degradation products.

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